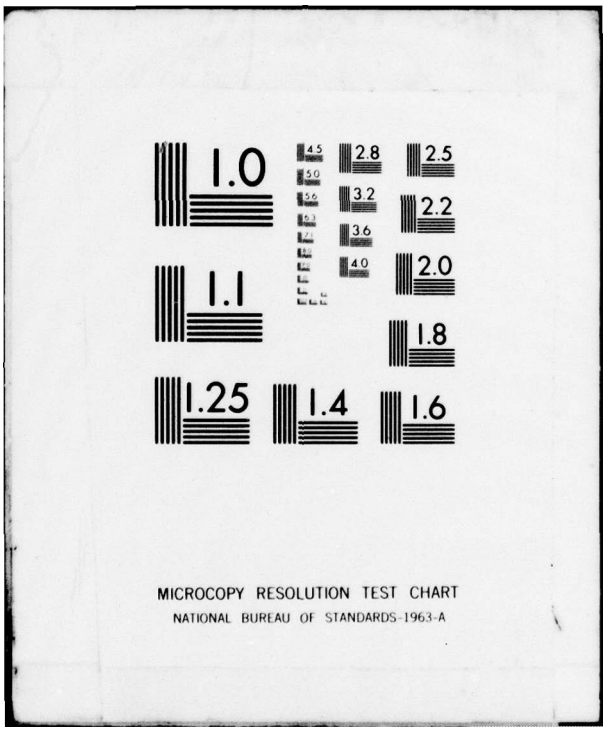
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Volume I.

(PROJECTS AND WORK UNITS ARE LISTED IN TABLE OF CONTENTS)

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ANNUAL PROGRESS REPORT.
1 OCTOBER 1977 — 30 SEPTEMBER 1978.

VOLUME I

743 p.

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INCLUDING

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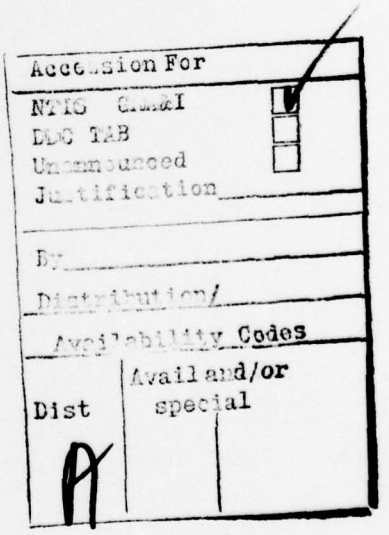
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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.



SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of the investigators are given at the beginning of each report.

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Project 3A161101A91C

IN-HOUSE LABORATORY INDEPENDENT RESEARCH

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☐ YES ☒ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	095
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Investigations of the Owl Monkey as a Laboratory Animal

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR — PRECEDING 78	1.5	70
FISCAL YEAR — CURRENT 79	1.5	80

19. RESPONSIBLE DOD ORGANIZATION

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ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Rapmund, Garrison, COL, MC

TELEPHONE: 202 576-3551

20. PERFORMING ORGANIZATION

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Washington, DC 20012

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TELEPHONE: 202 427-5430

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Aotus trivirgatus; (U) Owl monkey; (U) Breeding; (U) Karyotype

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To obtain biologic and physiologic data of the owl monkey essential for optimizing colony management procedures, recognizing disease states and breeding and raising Aotus for studies of falciparum malaria, for which it is the best nonhuman primate host. Aotus are no longer commercially available in sufficient numbers to meet the Army's requirements for its malaria drug development research program. Physiologic data, presently unavailable or unconfirmed for the owl monkey, is critically needed to assure the success of a domestic breeding effort to supply these monkeys for malaria research.

24. (U) Physiologic data will be obtained from infants to fully mature adults to include the following measurements: hematologic, serum biochemical and enzyme, estrous cycle, gestation period, and growth and development patterns. Standard procedures will be used.

25. (U) 77 10 - 78 09 During the year, there were 30 live births, 6 abortions, and 1 premature birth in the WRAIR Aotus colony. The death rate among newborn infants was approximately 50 percent, with Klebsiella pneumoniae being the principal pathogen identified. An attempt to hand rear infant Aotus free of K. pneumoniae, using a bioclean environment, was unsuccessful. The death rate for adult owl monkeys approximated 10 percent. Principal causes of deaths were associated with anemia, glomerulonephritis, and infections with K. pneumoniae, studies of which are in progress. Chorionic gonadatropin measurements for 5 pregnant Aotus confirmed previous estimates of an 18 week gestation period for owl monkeys. Serum assays for estrone, estradiol, and progesterone could not be used to define the estrous cycle. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Investigators:
Principal: LTC R. D. Hall, VC
Associates: MAJ M. J. Reardon, VC; LTC R. J. Beattie, VC; LTC T. J. Keefe, VC

Description:

To obtain biologic and physiologic data on the owl monkey (Aotus trivirgatus) which are needed to optomize colony management, recognize disease states, and successfully breed and rear these monkeys for falciparum malaria studies, for which it is the best nonhuman primate host.

During the reporting period, research activities have included analyses of: (1) specific parameters affecting reproduction, (2) rearing infant monkeys in a bioclean environment, (3) a hemagglutination inhibition test for early detection of pregnancy, and (4) serum assays for esterone, estradiol, and progesterone to define the estrous cycle.

1. Reproduction data.

Certain basic reproductive and survival data are essential for estimating the future productivity of a breeding colony. This information is particularly necessary for setting production goals, estimating basic colony size to meet those goals, estimating production costs, and planning colony management and support activities. These data are not available in the literature for the owl monkey (Aotus trivirgatus). To develop this information for owl monkeys, studies were made of a colony of 94 breeding pairs of monkeys. Preliminary data were presented previously (16) and were expanded during the past year.

Of the original 94 pairs of monkeys, 57 pairs (61%) survived the 2 year period, 9 pairs (9%) were separated because of fighting and 28 pairs (30%) were terminated by death of one or both of the breeders. Thirty-four of the original 188 monkeys died during the study, giving an average annual death rate of 9%. Analysis of the causes of death of adult owl monkeys is

provided in a separate section of this report.

Female breeder monkeys could be segregated into 3 distinct groups based on their reproductive performance: 47 females (50%) produced 60 single, viable offspring; 16 females (17%) became pregnant, several on multiple occasions, but all of these pregnancies ended in miscarriages; and 31 females (33%) never conceived during the 2 years. In the group that never conceived, 8 pair were separated because of fighting and 13 pair were separated by death of one of the members. Ten of the females remained with their mates the entire 24 months.

Pregnancies were diagnosed when at least 3 successive urine specimens from a breeding pair showed a positive reaction with the Subhuman Primate Pregnancy Test kit (Section 3, below). A total of 113 pregnancies were detected. Of these 46 (41%) ended in spontaneous abortion, 7 (6%) ended in stillbirths, and 60 (53%) ended with single, live births. Four stillbirths and 16 abortions occurred among 15 females which produced at least one live offspring each. The remaining 3 stillbirths and 30 miscarriages occurred in the 16 female monkeys that never produced live offspring. Abortions usually occurred without overt signs. Most of the pregnancies that terminated early by abortion probably would have gone undetected without the use of the pregnancy test kit.

At the end of the 2 year study period, only 33 of the 60 offspring born (55%) were still alive. Summarization of the clinical and pathological data on these infants is not complete; however, a preliminary impression is that septicemia due to Klebsiella pneumoniae was the major cause of death. Studies are presently in progress to develop methods of reducing the death rate of infant owl monkeys. Other factors such as karyotypes and age are still being analyzed for their effect on the reproductive performance of these 94 pairs of owl monkeys.

2. Rearing infant owl monkeys in a bioclean environment.

The prevalence of Klebsiella pneumoniae among the owl monkeys in the WRAIR colony has significant implications on the health of the monkeys and their subsequent usage for breeding or investigations. The role of K pneumoniae may be either as a primary or a secondary pathogen. Among the adult owl monkeys in the colony, this organism has been isolated from tissues of monkeys that died from anemia, glomerulonephritis, and air sacculitis. Isolations of K pneumoniae also were made from throat and/or rectal swab specimens of more than 50% of the

monkeys examined (detailed analysis is provided in a separate section of this report). Similarly, the death rate among infant owl monkeys born in the WRAIR colony has been approximately 50%, with the most common cause of death being attributed to K pneumoniae infections.

Continued operation of an owl monkey breeding colony is predicated upon eliminating K pneumoniae from the monkeys within the colony. Further, malarial and other research investigations are apt to be compromised in the absence of pathogen-free owl monkeys. An attempt was made, therefore, to isolate neonatal owl monkeys from their parents and hand rear the infants under controlled environmental conditions.

Four newborn owl monkeys were separated from their parents within 24 hrs after birth. Each infant was washed thoroughly for about 15 min using an iodinated surgical soap (Betadine, Bard Pharmaceuticals, Inc), and transferred to an individual cage within an 8 ft X 12 ft, flexible wall Bioclean unit (Fieldstone Corp). The Bioclean unit was designed to minimize aerosol transmission of microorganisms by providing large volumes of highly filtered air to the interior in a vertical laminar flow. Personnel entering the Bioclean unit were required to wear sanitized foot coverings and sterile surgical gowns, caps, masks, and gloves. Equipment and materials taken into the unit were either sanitized or sterilized. Fecal specimens from each infant were collected from the cage and swabs of the equipment within the unit were examined culturally for K pneumoniae on a weekly basis.

The first infant owl monkey, I-1, was placed into the Bioclean unit on 16 March 1978 (day 0), and the second infant, I-2, was introduced 7 days later. Fecal samples from I-1 were negative for K pneumoniae on days 7 and 14, as was a fecal sample from I-2 on day 14. Between days 14 and 21, infants I-3 and I-4 were placed in the unit, both on the same day. Fecal samples on day 28 from all 4 infants yielded K pneumoniae. The source of this contamination undoubtedly was I-3, I-4, or both. Since each infant had been thoroughly cleansed prior to transfer into the Bioclean unit, the contaminating Klebsiella organisms probably were sequestered in the oral-pharyngeal area or in the gastrointestinal tract. The most likely means of dissemination of the organisms among the infants within the unit was via feeding and handling. The 4 infants were removed from the Bioclean unit after Klebsiella infection was detected. One of the infants died subsequently of a septicemia caused

by K pneumoniae. The remaining 3 infants have lived and continue to be observed for clinical signs of disease.

Although this initial attempt to rear Klebsiella-free owl monkeys was unsuccessful, the findings of the study provided the basis for establishing improved methods for management of the Aotus breeding colony. Changes made in breeding management of the owl monkeys include: (1) bacteriologic examination for Klebsiella sp of all breeder monkeys to define those that are Klebsiella-free, (2) segregation of infant monkeys followed by bacteriologic evaluation, (3) transfer of only Klebsiella-free infants into a clean rearing room or Bioclean unit, and (4) require personnel to change into clean gowns and gloves before handling each infant to reduce the potential of cross-contamination. Strict adherence to these principals, it is felt, will significantly enhance the quality of owl monkeys reared in the colony and result in a marked reduction of the infant mortality.

3. Pregnancy diagnosis in owl monkeys: Evaluation of the hemagglutination inhibition test for urinary chorionic gonadotropin.

Restrictions limiting the supply of feral New World monkeys for biomedical research have hastened the need for domestic breeding programs. The unique status of the owl monkey (Aotus trivirgatus) as a primate model for research on human malaria prompted the establishment of a colony of owl monkeys at the Walter Reed Army Institute of Research (WRAIR). An important management problem encountered was pregnancy diagnosis. Initially diagnosis was made by uterine palpation; however, this procedure required frequent handling of the monkeys which may have contributed to the occurrence of abortions and stillbirths. A reliable diagnostic method was sought that would detect pregnancy as early as possible without physical manipulation of the monkeys.

The Subhuman Primate Pregnancy Test (SPPT) kit has been used for detecting pregnancy in macaques (1,2,3), baboons (1,4), marmosets (5), tamarins (6), squirrel monkeys (7), chimpanzees (1,8,9), orangutans (9,10,11), and gorillas (1). This hemagglutination inhibition tube test for urinary chorionic gonadotropin uses an antiserum (H-26) to the β-subunit of ovine luteinizing hormone which cross-reacts with the chorionic gonadotropins of a variety of primate species and provides definitive results within two hours using neat urine. In addition, this same antiserum is employed in a radioimmunoassay

(RIA) system for quantification of chorionic gonadotropins (12).

The purposes of this study were to define the pattern of excretion of urinary chorionic gonadotropin during gestation of the owl monkey and to evaluate the application of the SPPT kit to detecting pregnancy in owl monkeys using a practical urine collection technique.

Animals and management. Monkeys (36 pairs) were housed as breeding pairs in galvanized, wire mesh cages measuring 0.75 X 0.75 X 0.75 m. Monkey rooms were maintained between 20 and 26 C with approximately 15 air changes per hour of 100% fresh air. Relative humidity was not controlled. Subdued lighting, obtained with fluorescent light tubes covered with red polycarbonate shields, was provided from 0600 to 1800 hours and was followed by darkness. Monkeys were fed a commercial, 25% protein, primate diet supplemented daily with apples or oranges.

Urine collection. In the initial evaluation phase of this study, urine was collected weekly from female monkeys expected to become pregnant. The females were separated from their cagemates during collection procedures with plexiglass partitions placed into the cages. Midmorning urine, aspirated from plastic sheets arranged under the female's side of the cage, was divided into two aliquots: 1 for immediate testing with SPPT, and the other stored at -20 C for later testing by RIA. When the SPPT kit was being evaluated for routine, colony-wide use, urine was collected biweekly from 31 breeding pairs, including pregnant females, without separating the monkeys. The danger of collecting female urine diluted with male urine, or male urine alone, is recognized. In both phases of this study, when a SPPT result changed from consistently negative to positive or equivocal (±), frequency of urine collection was increased to 2 or 3 times per week until a definitive pattern of positive or negative responses was established.

Testing procedure. All qualitative tests for Aotus chorionic gonadotropin (AoCG) were performed at the WRAIR using the SPPT kit according to previously described procedures (2). Coded urine specimens from five pregnant female monkeys were sent to the National Institutes of Health (NIH) for the quantitative assay for AoCG using RIA as previously described (12). SPPT results were not made available to NIH personnel until testing was completed.

Evaluation of the SPPT kit. During this phase of the study,

5 monkeys became pregnant and delivered live offspring. Urine specimens collected throughout these pregnancies were assayed by the SPPT kit and RIA for AoCG. Mean concentrations of AoCG at various stages of pregnancy are shown in Figure 1. The RIA technique detected urinary chorionic gonadotropin 16 weeks before parturition in all pregnant owl monkeys. After that, concentrations of AoCG increased, reaching a zenith 6 to 11 weeks before birth. About the 6th week prepartum the concentrations of AoCG declined, but clearly were detected until parturition. Within 1 week post partum, AoCG could not be detected in urine.

Results of the hemagglutination inhibition test for AoCG using the SPPT kit on these same urine specimens are also shown in Figure 1. Between the 16th and 14th week prepartum, only half of the specimens showed a positive reaction. However, between the 14th week and the last week, the SPPT results were highly reliable for predicting pregnancy, exceeding 90% accuracy. Equivocal (±) results bracketed or followed by a series of at least 3 positive reactions were considered positive.

These equivocal results were seen most frequently at the beginning and end of the gestation period when chorionic gonadotropin levels were near the limits of detection in the SPPT.

Routine application of the SPPT. SPPT results were analyzed for a group of 62 owl monkeys which remained paired for 24 months. Data in Table 1 show the frequency of correct and incorrect test responses on 1688 urine specimens from these monkeys. Seventeen of the 31 females produced 21 single, viable offspring during the 24 months. The other 14 females did not give birth during the 2 years. Apparently, 9 of the nonparous females never conceived during the study period. An additional 5 females are known to have become pregnant a total of 16 times, yet fetal loss occurred prematurely. Abortions usually occurred without overt signs. On only 2 occasions was the fetus or fetal material observed after a spontaneous abortion.

A correct negative or positive pregnancy test response was observed 88% of the time (Table 1). False negative and false positive reactions were observed 2 to 5% of the time, and equivocal (±) or inconclusive test responses were observed 5 to 8% of the time.

These results demonstrate that the SPPT kit is useful for pregnancy diagnosis in owl monkeys as early as 14 weeks before par-

turition. When a test result indicates a change from a consistently negative pattern to positive or equivocal, the overall accuracy of the SPPT is greatly increased by performing a second independent confirmatory test. In this study, false negative results were probably caused by testing or dilution with male urine. Reasons for positive and equivocal results when a monkey is not pregnant are presently under study. By testing urine from breeding females biweekly, a high degree of accuracy, at least 90%, can be obtained by predicting parturition 100 $\pm$ 10 days from the first confirmed positive SPPT result.

Detecting early abortions by monitoring urinary chorionic gonadotropin levels is essential information when forming a reproductive profile for breeder owl monkeys. Data from this study show that owl monkeys continue to produce AoCG at detectable levels until the week of delivery. This makes the SPPT particularly useful for monitoring continuation of gestation in owl monkeys. The SPPT kit allowed us to detect spontaneous abortions rapidly and accurately, and eliminated the need for frequent handling to perform uterine palpation, which, itself, may contribute to fetal wastage.

The Subhuman Primate Pregnancy Test (SPPT), a hemagglutination inhibition tube test, was evaluated as a means of detecting urinary chorionic gonadotropin (UCG) and related pregnancy in owl monkeys. Using radioimmunoassay (RIA), the excretion pattern of UCG from pregnant owl monkeys was delineated, the hormone being detected from 16 weeks prepartum until birth. By comparison, the SPPT detected UCG and pregnancy between week 14 prepartum and the last week of gestation with greater than 90% accuracy. In a 2 year study using a simplified urine collection technique, the SPPT was shown to be a valuable procedure for diagnosing pregnancy and detecting spontaneous abortions in owl monkeys.

4. Electrophoretic study of serum proteins in karyotypically defined owl monkeys.

Serum protein polymorphism, to include bisalbuminemia and triple alpha globulinemia, has been observed in owl monkeys by several investigators (13,14,15). The number of animals examined, however, usually has been small and interpretations of the results have conflicted. The serum immunoelectrophoretic patterns reported for 5 monkeys showed Brazilian (ikaryotype I) owl monkeys had 2 distinct albumin fractions, whereas Colombian owl monkeys only exhibited one (14). By contrast, 0 of 5 karyotype

I and 2 of 6 Colombian monkeys included in another study had bisalbuminemia (15). This 2 albumin fraction phenomenon was substantiated when bisalbuminemia was detected in 33 of 110 owl monkeys; however, the relationship of these findings to karyotype was not studied (13). A second serum protein polymorphism, expression of a third macroglobulin, also has been reported for some owl monkeys. Collins et al observed 3 alpha globulins in each of 5 karyotype I monkeys and only 2 alpha globulins in each of 6 Colombian monkeys. Additionally, it has been shown that karyotype I owl monkeys have increased resistance to infection with Plasmodium falciparum, while the remaining 6 karyotypes, although variable in response, are susceptible. Expression of these 2 polymorphisms by some owl monkeys and not others suggested that specific serum electropherograms possibly could be associated with given karyotypes. Consequently, definition of the electrophoretic pattern for each owl monkey karyotype would provide a rapid and simple means of ascertaining the karyotype of individual monkeys, as well as provide an indicator system of susceptibility of each individual to malarial infection. The purposes of this study were to: (1) determine the serum protein composition for owl monkeys with known karyotypes, (2) examine the correlation of specific electropherograms with one or more karyotypes, and (3) analyze the genetic basis for serum polymorphisms in owl monkeys using data obtained for adult monkeys and their offspring. Preliminary data previously reported were incorporated into this study (16).

Sera from 238 owl monkeys of known karyotype were examined by cellulose acetate electrophoresis using the Gelman Sepratek system. Marker animals were selected to use as standards for comparison.

Bisalbuminemia was observed for 75 of the monkeys, while the remaining 163 monkeys had only 1 albumin fraction. Examination of the electropherograms for the monkeys exhibiting single albumin fractions showed that migration of the albumin for 109 of the monkeys corresponded to the albumin fraction of bisalbuminemic monkeys with the faster electrophoretic mobility (designated fast albumin). Migration of the albumin of the remaining 54 monkeys coincided with the mobility of the slower albumin fraction of the bisalbuminemic monkeys. The total concentration of serum albumin for monkeys with bisalbuminemia was similar to the concentrations detected for monkeys having a single albumin fraction. Concentrations of the fast and slow albumins in bisalbuminemic monkeys were approximately equal.

Distribution of the serum albumin phenotypes among the differ-

ent karyotypes is shown in Table 2. Single fast or slow albumin fractions or both fractions were observed among 222 owl monkeys of karyotypes I, II, III, or IV. Of these monkeys, 109 (49%) only had fast albumin and 38 (17%) only had slow albumin. Bisalbuminemia was detected in 75 of the 222 (38%) monkeys, and occurred in monkeys of karyotypes II, III, and IV. The absence of bisalbuminemia among monkeys of karyotype I was an unexpected finding since the fast and slow albumins occur independently. Examination of progeny derived from mating fast albumin karyotype I monkeys with slow albumin monkeys should clarify this discrepancy, as the offspring would be expected to have both albumin fractions. A total of 16 monkeys of karyotypes V, VI, and VII expressed only the slow albumin. Electrophoretic separation of serum proteins of progeny derived from various matings demonstrated that albumin polymorphism was controlled genetically in owl monkeys (Table 3). These data are consistent with similar observations made previously for man (17) and other species (18) where bisalbuminemia has been shown to be related to expression of codominant autosomal alleles.

Three alpha globulins were observed in 29 monkeys of karyotypes I and VII (Table 2). The remaining 209 monkeys (karyotypes II, III, IV, V, VI) had only the 2 commonly defined alpha globulins. The third globulin exhibited faster electrophoretic mobility than the other 2. Using standard nomenclature, the 2 common alpha globulins are designated alpha 1 (fast) and alpha 2 (slow); therefore, the third alpha globulin was termed "fast alpha" in lieu of "alpha 3" by virtue of its electrophoretic mobility.

Each of the 29 owl monkeys exhibiting fast alpha globulin were of karyotypes I and VII. None of the monkeys of the other 5 karyotypes exhibited this trait. Four hybrid progeny were obtained by mating monkeys of karyotype I with monkeys of karyotypes II, III, V, and VII. All 4 hybrid progeny expressed fast alpha globulin. Although the number of animals was small, these observations suggested that expression of fast alpha globulin was a dominant genetic trait.

Although albumin and alpha globulin polymorphisms occur with different frequencies among the owl monkey karyotypes, the karyotype of an individual monkey can not be ascertained by serum electrophoresis. Expression of fast alpha globulin may prove to be of value in predicting the susceptibility of individual monkeys to malarial infection. Confirmation of this potential, however, must await completion of additional serum electrophoretic studies of hybrid monkeys, followed by subsequent infectivity analyses.

5. Definition of the estrous cycle of owl monkeys.

Different methods of analysis have been employed to define the length of the estrous cycle in owl monkeys (16,19). Examination of vaginal smears obtained daily for cytologic alterations indicated the length of the cycle to be 14 ± 4 days (19). The data were inconclusive, however, since only 13 cycles were observed in 28 female monkeys over a 1 year study period. Difficulty in recognizing the different estrous cycle stages principally was attributable to the minimal alterations Aotus vaginal epithelium undergoes during estrous as compared with other species (19). In a second study, serum estrogen levels were quantified and plotted for 5 adult, female monkeys over a 2 month period (16). Sera were obtained from each monkey twice or thrice per week and were assayed for esterone and estradiol by radioimmunoassay. One of the 5 monkeys had alternating high and low levels of esterone and estradiol at periodic intervals, indicating the occurrence of regular estrous cycles. The time intervals between 4 of the estradiol peaks was 17, 17, and 14 days. Further attempts were made to extend and confirm these observations by quantifying and plotting serum progesterone levels in female owl monkeys.

In the initial study, serum samples were obtained from 6 female owl monkeys twice per week for 2 months. One of the monkeys was the female that had exhibited marked cyclical changes in esterone and estradiol concentrations (16), while the remaining 5 monkeys had never been used for test purposes. Sera were assayed for progesterone by radioimmunoassay (performed at National Institute of Child Health and Human Development, Bethesda, MD). Two of the 6 monkeys had cyclical changes in progesterone levels, with concentrations less than 10 ng/ml at the nadir followed by 40 to 80 ng/ml at the zenith. Time intervals between peak values ranged from 5 to 14 days. The mean for 12 time intervals was 9 ± 3 days. These data, therefore, suggested the duration of the estrous cycle for owl monkeys was significantly less than observed previously (16,19). Progesterone levels measured for the remaining 4 monkeys were erratic, precluding interpretation.

A second study was initiated after the observation of a 9 day estrous cycle, the purposes of which were to evaluate the daily changes in progesterone levels and to further define the length of the estrous cycle. Sera from 6 female monkeys were obtained daily for 10 to 17 days, and were assayed for progesterone by radioimmunoassay (performed by Hazelton Laboratories, Inc., Vienna, VA). Again, a broad range was observed with a

low of 3 ng/ml to a high of 244 ng/ml. Two of the monkeys had progesterone levels in excess of 200 ng/ml in each of 3 successive serum samples. Although a complete cyclical pattern was not observed for any of the 6 monkeys, definite upward and/or downward trends were noted. The absence of definitive estrous cycles undoubtedly was attributable to the brevity of the duration of sampling and the asynchrony of estrous among the monkeys examined.

The levels of progesterone observed for the owl monkeys were higher than was expected because serum concentrations in Old World monkeys during the luteal phase of the cycle only attain a level of 5 to 8 ng/ml (20). The principal limitation to studies requiring serum analyses is the volume of blood that can be withdrawn at each interval and over a prolonged period from an owl monkey without causing detrimental effects. The size of owl monkeys places strict constraints on this limitation. Thus, only one steroid assay can be accomplished with the serum volume obtainable at each bleeding interval. Preferably assays for estrogens, progesterone, and luteinizing hormone would be performed using the same serum samples. Frequent handling may result in alterations or deviations in the normal estrous cycle, which would account for the few monkeys exhibiting complete estrous cycles throughout these investigations and each of the 2 previous studies (16,19). Consequently, the number of monkeys included in any study of the estrous cycle must, by necessity, be at least 3-fold greater than the needed number of monkeys exhibiting cyclical patterns.

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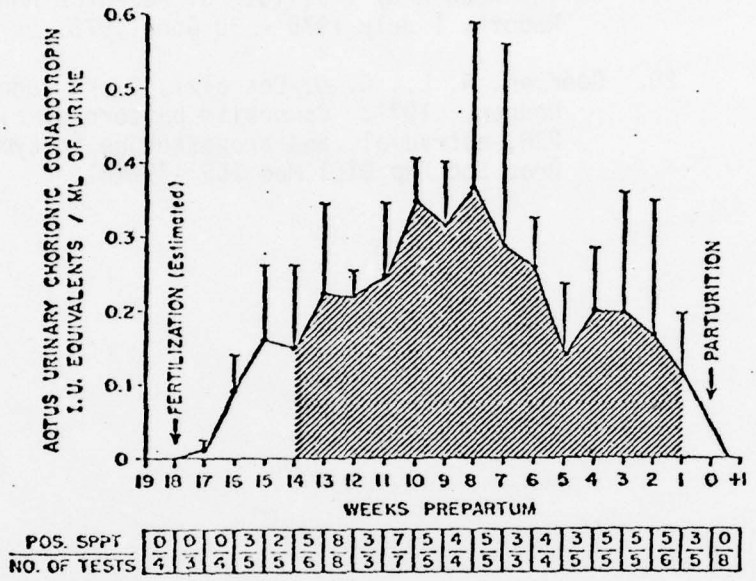


Fig 1. Patterns (Mean + 1 S.D.) of chorionic gonadotropin excretion in urine of five pregnant owl monkeys as measured by radioimmunoassay. Shaded area illustrates interval over which 94% of all SPPT responses were positive. Fractions (at bottom) indicate the number of positive SPPT results over the total number of specimens tested each week throughout pregnancy. The estimated time of fertilization was based on preliminary observations made during a vaginal cytology study (Hall, unpublished). I.U. equals one International Unit of Human Chorionic Gonadotropin.

Table 1. Results of Hemagglutination Inhibition Tests for Urinary Chorionic Gonadotropin in Pregnant and Non-pregnant Owl Monkeys.

Females	Number of animals	Number of tests	Test response: Correct (%)	False negative (%)	False positive (%)	Equivocal (±) in non-pregnant females (%)[a]
Term deliveries[b]	17	888	790 (89.0)	32 (3.6)	23 (2.6)	43 (4.8)
Spontaneous abortions[c]	5	376	326 (86.7)	7 (1.9)	20 (5.3)	23 (6.1)
Nulliparous[d]	9	424	375 (88.5)	NA[e]	15 (3.5)	34 (8.0)
	31	1688	1491 (88.3)	39 (2.3)	58 (3.5)	100 (5.9)

[a]Equivocal responses during pregnancy were considered positive (correct) responses.

[b]Females which gave birth to live offspring during the 24 month study period.

[c]Females which conceived but pregnancies terminated in abortion or resorption and no births occurred during the study period.

[d]Females which never conceived during the study period.

[e]Not applicable.

Table 2. Occurrence of Fast, Slow, or Double Albumin Fractions and Fast Alpha Globulin Among Owl Monkeys as Distributed by Karyotype and Sex

Karyotype	Sex	Number tested	Albumin (%)						Fast alpha globulin
			Fast only		Double		Slow only		
I	Male	18	15	(83)	0	(0)	3	(17)	18
	Female	10	6	(60)	0	(0)	4	(40)	10
II	Male	43	21	(49)	14	(33)	8	(18)	0
	Female	38	19	(50)	15	(39)	4	(11)	0
III	Male	40	15	(38)	19	(48)	6	(14)	0
	Female	47	20	(43)	19	(40)	8	(17)	0
IV	Male	11	5	(45)	4	(36)	2	(19)	0
	Female	15	8	(53)	4	(27)	3	(20)	0
V	Male	1	0	(0)	0	(0)	1	(100)	0
	Female	2	0	(0)	0	(0)	2	(100)	0
VI	Male	6	0	(0)	0	(0)	6	(100)	0
	Female	6	0	(0)	0	(0)	6	(100)	0
VII	Female	1	0	(0)	0	(0)	1	(100)	1

Table 3. Distribution of Serum Albumin Phenotypes Among 38 Progeny from Various Matings of Owl Monkeys

Phenotype of parents	Phenotype of progeny		
	FF[a]	FS[b]	SS[c]
FF X FF	9		
FF X FS	7	10	
FF X SS		1	
FS X FS	1	4	1
FS X SS		2	2
SS X SS			1

[a]FF = fast albumin fraction

[b]FS = double albumin, both fast and slow albumin fractions

[c]SS = slow albumin fraction

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6448	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	096
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Biochemical and Immunological Characterization of Rickettsial Proteins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	4	141
FISCAL YEAR CURRENT 79	4	163

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Robinson, David, LTC, VC
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NAME: Eisemann, Christine

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Biochemistry; (U)Structure-Function Relationship; (U)Structure-Antigenicity Relationship

23. TECHNICAL OBJECTIVE 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) 1. Isolation and purification of subcellular components responsible for eliciting immunological protection; 2. Localization and identification of surface proteins that affect virulence; 3. Antigenic characterization of peripheral proteins. These studies will aid in identification of rickettsial strains and in the development of inactivated vaccines capable of protecting troops in the field.

24. (U) 1. Utilization of peripheral rickettsial macromolecules as experimental immunogens. 2. Rickettsial purification by sucrose or potassium tartrate gradient centrifugation or isoelectric focusing methods. Analysis of proteins by polyacrylamide gel electrophoresis and immunoelectrophoresis with specific radiolabeling by extrinsic radiodination or sodium borohydride reduction. 3. Determination of the antigenic interrelationships between strains of scrub typhus rickettsiae by a plaque reduction, complement fixation, immunofluorescent, cross-neutralization, and mouse virulence tests using specific anti-rickettsial antibody prepared from plaque-purified scrub strains.

25. (U) 77 10 - 78 09 1. Six proteins, previously established as major constituents of intact organisms were identified in cell envelopes of R. prowazekii. Two proteins, No. 4 and No. 1, were most peripheral and probably interact with the immune system of the host. 2. Extrinsic radioiodination failed to selectively label components of the cell wall of R. conorii suggesting cell walls of spotted fever organisms have a different antigenic topography than the typhus group. 3. Extraction of soluble antigen from typhus group rickettsiae indicated the presence of all proteins except No. 2, which remains associated with particulate rickettsiae and may contribute to the species-specific CF antigen. The plaque reduction test has been developed and initial homologous and heterologous plaque reduction tests are complete. Results indicate the test to be useful for strain characterization and comparisons. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 096 Biochemical and Immunological Characterization of Rickettsial Proteins

Investigators.

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Associates: Christine S. Eisemann; Janis Campbell; SP5 John Dewitt; SP4 Matthew Nypaver

Description.

The peripheral rickettsial macromolecules are being investigated to characterize subcellular components potentially important in establishing rickettsial infection or stimulating an immunological response in the host, to study the relationships of the rickettsiae, to develop new serological tests for diagnosing rickettsial disease and to assess host protection to infection. Also, a plaque reduction test has been developed which has the potential for discriminating between strains of scrub typhus rickettsiae.

Progress.

I. Surface proteins of typhus and spotted fever group rickettsiae.

The proteins of intact rickettsiae have been analyzed by electrophoretic techniques, and the established profiles have been used for identification and comparison of different species of rickettsiae (1). The importance of these proteins to the disease process, including the establishment of infection and the immune response of the animal host, is likely to be dependent upon their morphological location in the organism. Surface proteins, especially those on the periphery of the rickettsial cell envelope, are most accessible to the immune system of the host and probably constitute the major antigenic stimulus for a protective humoral or cellular response. The cell envelope of both typhus group and spotted fever group rickettsiae is the source of soluble antigen (2), (a peripheral, group-specific antigen important in rickettsial serology). Polyacrylamide gel electrophoresis (PAGE) of soluble antigen obtained from R. rickettsii indicated the antigen was complex and contained at least 9 proteins (2). Further studies on peripheral proteins are lacking, and without reliable identification of rickettsial cell envelope proteins and analysis of their topographical

relationship, it is difficult to hypothesize their role in rickettsiae-host cell interactions. The purpose of this study was to identify peripheral proteins by two different approaches: 1) isolation of cell envelopes and analysis of intrinsically radiolabeled proteins, and 2) selective peripheral labeling of intact organisms by extrinsic radioiodination.

A. Preparation and analysis of cell envelopes.

Intact rickettsiae intrinsically radiolabeled with adenine and amino acids banded uniformly in a potassium tartrate density gradient (Fig. 1a). Cell envelopes, prepared by disruption in a French pressure cell were less dense than intact organisms and were devoid of adenine (Fig. 1b). Physical differences between intact rickettsiae and cell envelope preparations were also evident in sucrose velocity gradients. Intact organisms banded uniformly, as previously reported (1), but cell envelopes were unable to enter a similar gradient and remained at the meniscus. Thus, the cell envelope fraction of R. prowazekii was easily distinguished from intact organisms by gradient centrifugation procedures, contained no detectable contaminating whole cells, and was used as an enriched source of peripheral proteins.

B. Protein composition of cell envelopes.

PAGE of intrinsically radiolabeled cell envelope showed a migration pattern very similar to intact rickettsiae (Fig. 2). The 6 major proteins of R. prowazekii, previously identified and characterized as components of intact organisms (1) were also prominent in the gel pattern of cell envelopes. These data established the morphological location of the major proteins, but the intrinsic labeling technique could not discriminate their relative location in the cell envelope. A selective peripheral labeling technique was required to identify the outermost proteins of the rickettsial cell envelope. Since radioiodination techniques often rely on substitution reactions involving tyrosine, we examined the 6 major proteins for the presence of this amino acid prior to initiating extrinsic labeling studies. The PAGE pattern obtained after intrinsic radiolabeling of R. prowazekii with tyrosine is shown in Fig. 3 and compared to the protein labeling obtained with the mixture of radioactive amino acids. Although there is a relative dimunition in tyrosine labeling of proteins 1 and 2, and a relative increase in tyrosine labeling of proteins 3 and 4, it was evident that none of the major proteins were either devoid of tyrosine or heavily enriched in this amino acid.

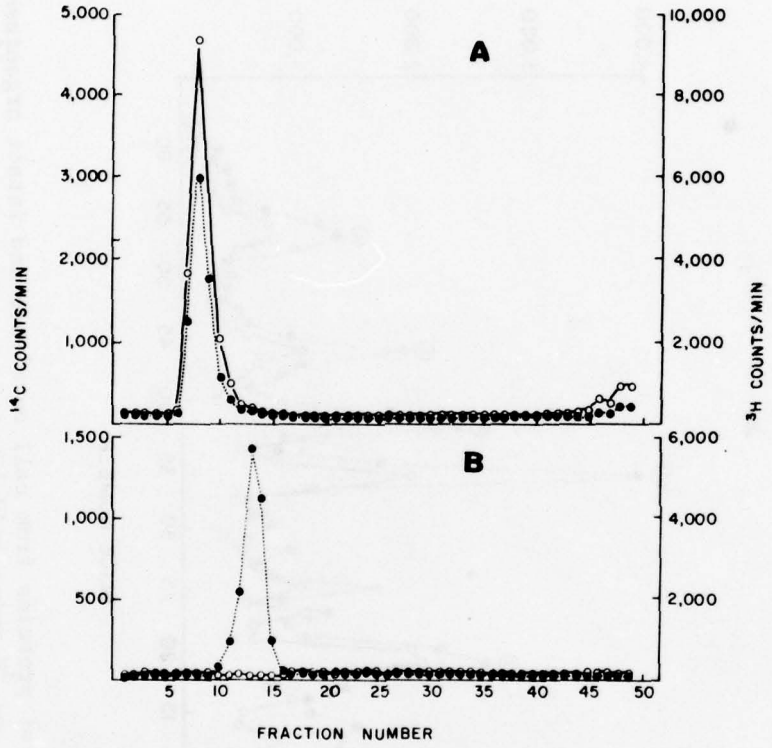


Fig. 1 Sedimentation of intact rickettsiae and cell envelopes in potassium tartrate density gradients. (a) intact R. prowazekii labeled with ^{3}H adenine (o——o) and ^{14}C amino acids (●----●); (b) cell envelopes prepared from dually labeled organisms.

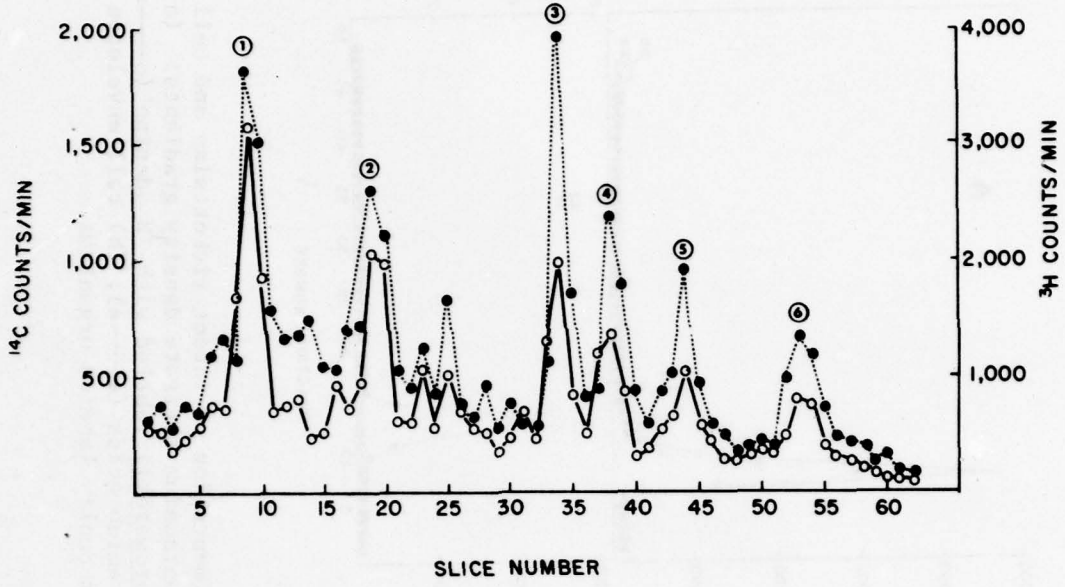


Fig. 2 Co-electrophoresis of proteins from cell envelopes and intact organisms of R. prowazekii. (o——o) ^{3}H amino acid labeled rickettsiae; (●----●) ^{14}C amino acid labeled cell envelopes.

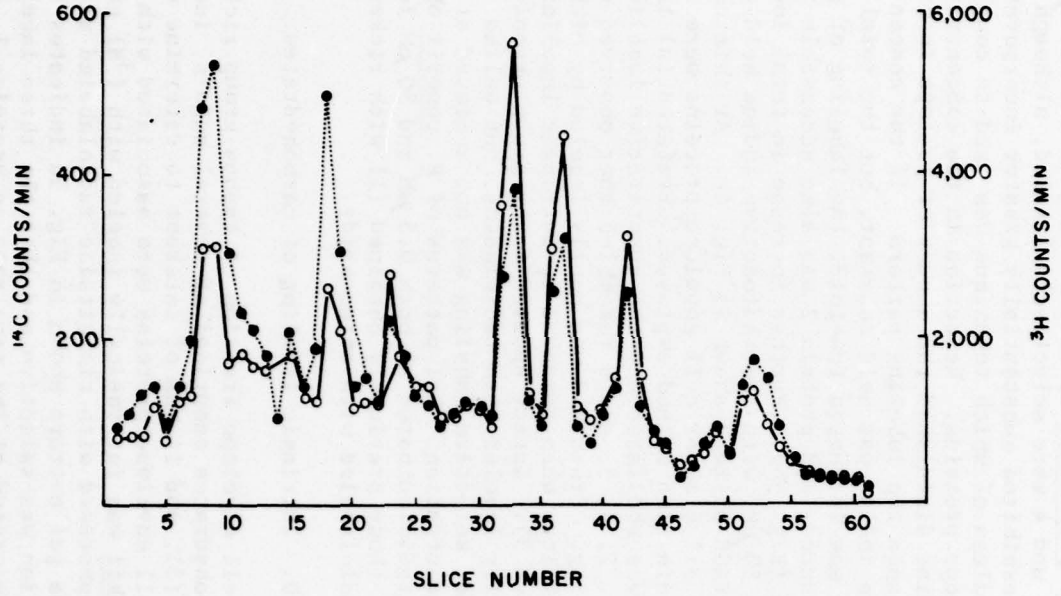


Fig. 3. Co-electrophoresis of R. prowazekii proteins labeled with tyrosine or amino acids. o——o, ^{3}H tyrosine; ●----●, ^{14}C amino acids.

C. Radioiodination of intact rickettsiae.

Rickettsiae were radioiodinated by either the chloramine-T or lactoperoxidase technique, sedimented in sucrose velocity gradients to remove free iodine, then examined by the PAGE procedure. Fig. 4a indicates the gel pattern observed after labeling R. prowazekii by both techniques with 0.5 μM radioiodine. Proteins 1 and 4 were selectively labeled, although the latter component exhibited substantially greater incorporation of isotope regardless of which technique was used to couple iodine to cell envelope proteins. Reduction in the concentration of radioactive iodine diminished the amount of isotope incorporation, but did not change the labeling pattern. If the concentration of radioactive iodine was held constant, but the total iodide concentration was increased ten-fold, the labeling of protein 1 was more pronounced, and protein 2 was also accessible for radioiodination (Fig. 4b). Further increase in total iodide concentration to 50 μM, with the radioactive iodine held constant, produced the PAGE pattern shown in Fig. 4c. At this concentration of iodide, all 6 major cell envelope proteins were labeled regardless of iodination method employed. Preferential labeling of protein 4 was abolished and the quantitative labeling pattern of proteins 1, 2, 4, 5, and 6 resembled that observed with intrinsic radiolabeling. Protein 3 was poorly labeled by either radioiodination technique when compared to intrinsic labeling patterns (Figs. 2 and 3). Intact spotted fever group organisms were also extrinsically labeled with radioiodine, but unlike the typhus rickettsiae, selective labeling was not achieved at the lower iodine concentration. Gel patterns of R. conorii obtained with organisms radioiodinated at both 0.5 μM and 50 μM iodide were similar to those previously obtained (1) with rickettsiae intrinsically radiolabeled with amino acids.

D. Intrinsic labeling of carbohydrates.

The cell envelope fraction of typhus group rickettsiae contains carbohydrates comprised of several sugars, including galactose (3), and it was of interest to determine which of the 6 major cell envelope proteins were associated with carbohydrates. R. prowazekii was intrinsically labeled with (^{3}H) galactose and co-electrophoresed with rickettsiae radiolabeled with ^{14}C amino acids. The gel pattern shown in Fig. 5a indicates that galactose incorporation was selective and that the three labeled macromolecules migrated at the same rate as proteins 1, 3, and 4. R. conorii was also intrinsically radiolabeled with (^{3}H) galactose and analyzed by PAGE (Fig. 5b). The results were essentially identical to those observed with typhus rickettsiae; sugar labeling was restricted to 3 macromolecules which co-migrated with R. conorii proteins 1, 3, and 4.

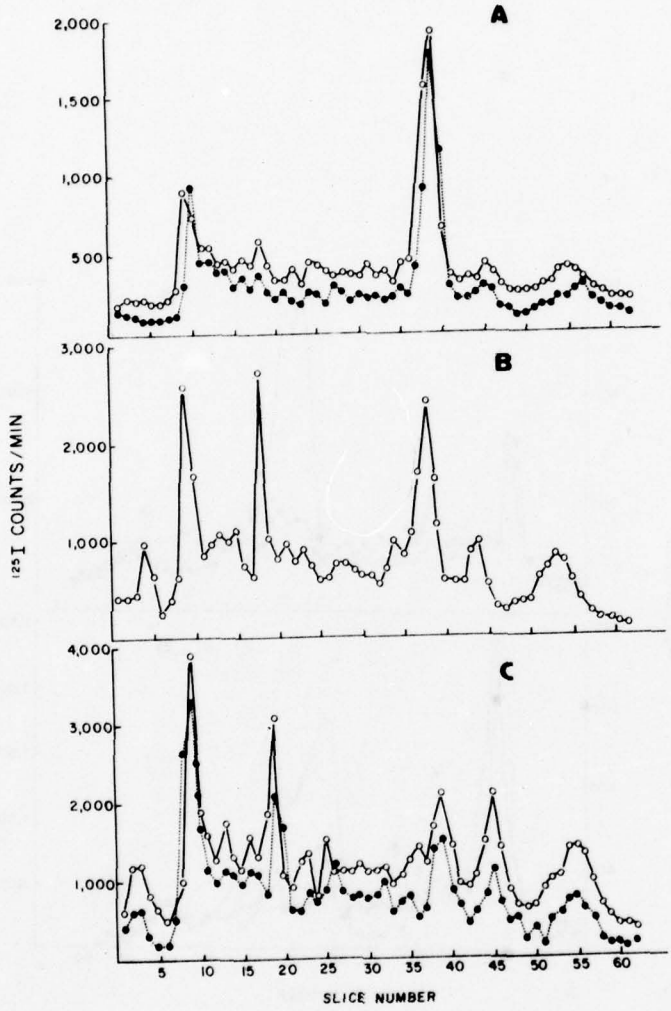


Fig. 4 Radioiodination of intact R. prowazekii. (a) 0.5 μM radioiodine; (b) 0.5 μM radioiodine plus 4.5 μM iodide (c) 0.5 μM radioiodine plus 49.5 μM iodide. o——o, chloramine-T technique; ●----●, lactoperoxidase technique.

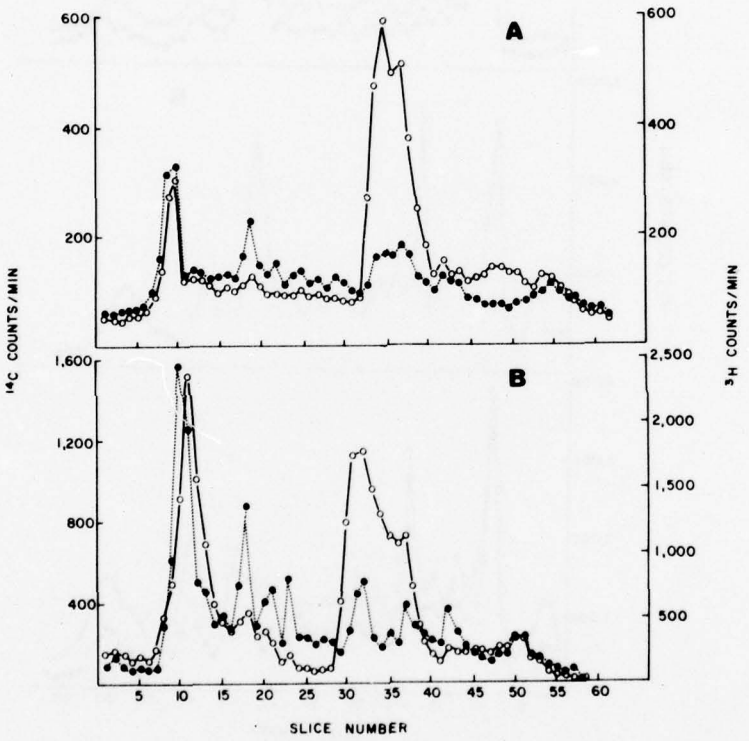


Fig. 5 Co-electrophoresis of intact rickettsiae labeled with galactose or amino acids. (a) R. prowazekii: o——o ^{3}H galactose; ●----●, ^{14}C amino acids; (b) R. conorii: o——o, ^{3}H galactose; ●----●, ^{14}C amino acids.

E. Soluble antigen.

Intact typhus rickettsiae, labeled with 0.5 μM radioiodine at a final iodide concentration of 50 μM were extracted with diethyl ether and the soluble antigen examined by PAGE. Fig. 6 indicates that the soluble antigen contained 5 of the 6 major cell envelope proteins. Protein 2 was not detected in soluble antigen, although gels of the residual corpuscular antigen remaining after ether extraction revealed this protein had been effectively labeled. This study clearly demonstrated that the 6 major proteins identified after PAGE of intact R. prowazekii (1) are located within the cell envelope fraction of the organism. Other workers have demonstrated that rickettsial cell envelopes constitute approximately 20% of the dry weight of intact organisms (3), and we anticipated that the principal components of intact organisms would also be prominent in the gel pattern of cell envelopes. The tyrosine content of each of these proteins was similar, and allowed the use of extrinsic radioiodination both to increase sensitivity of gel analyses and to explore the topographical relationship of cell envelope proteins. Radioiodination, performed by either the lactoperoxidase or chloramine-T procedures, yielded similar results at 0.5 μM iodide and established that protein 4 was fully accessible for labeling and was therefore likely to be the outermost protein in the cell envelope of R. prowazekii.

Minor labeling of protein 1 also occurred, indicating that some reactive sites were exposed on the surface of the rickettsiae. Incremental increases in iodide concentration resulted in other cell envelope proteins becoming reactive with radioiodine. More complete labeling of protein 1 and labeling of protein 2 was observed with a 10-fold increase in iodide concentration, while proteins 3, 5 and 6 were radiolabeled after a 100-fold increase in iodide concentration. We are not certain of the effect of iodide on the protein mosaic of the rickettsial cell envelope, but similar selective, concentration-dependent labeling has been achieved with Rous-associated virus and influenza virus. The influence of iodide concentration on the extent and specificity of radioiodine labeling by the chloramine-T method has been attributed to kinetic phenomena and the formation of concentration-dependent hydrophobic or hydrophilic iodinating species. The effect of iodide concentration on lactoperoxidase labeling has received less attention, although it is clear that low iodide concentration promoted peripheral labeling of enveloped viruses. In this study, as well as in a study of influenza virus, iodide concentrations equal to, or exceeding 50 μM affected the accessibility of proteins to the surface catalyzed enzyme labeling reaction mediated by lactoperoxidase. Regardless of whether

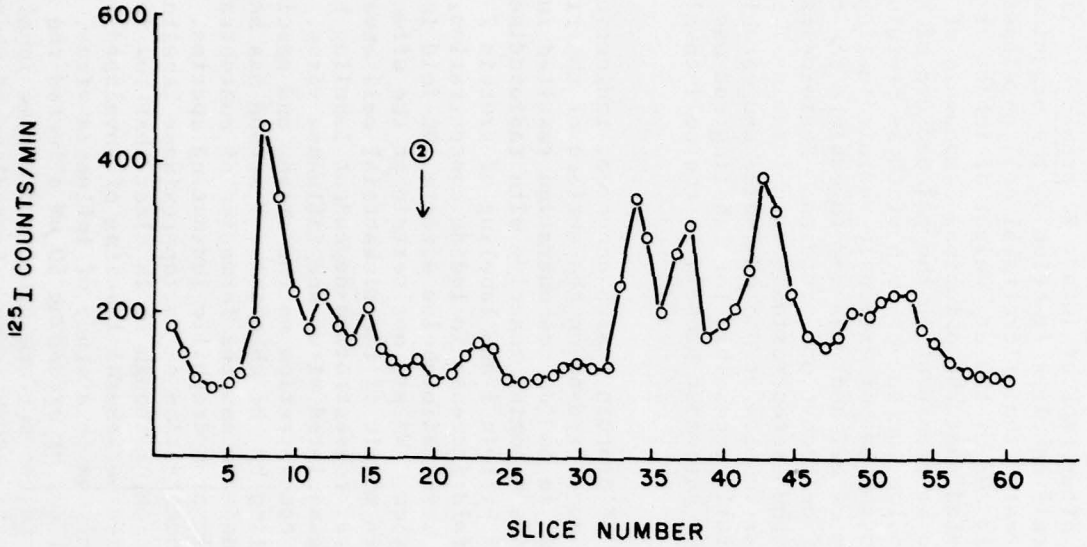


Fig. 6 Electrophoresis of ^{125}I radiolabeled soluble antigen obtained from R. prowazekii.

changes in rickettsial labeling were due to the increased hydrophilic nature of the reactive iodine complex, or due to perturbation of cell envelope morphology with increased accessibility of lactoperoxidase, we feel that the concentration-dependent iodide labeling technique provides a sensitive probe for determining the topographical orientation of cell envelope proteins. However, the specificity of labeling observed with R. prowazekii was not seen with the spotted fever group organism studied.

Over ten separate experiments were conducted to verify the uniform labeling of R. conorii at both 0.5 μM and 50 μM iodide. We feel that there is a fundamental difference in the array of proteins in the cell envelope of typhus and spotted fever group organisms. On the other hand, galactose labeling of rickettsiae produced uniform gel patterns with both typhus and spotted fever group organisms. Proteins 1, 3, and 4 appeared to be specifically associated with carbohydrates, although similar migration in gels is only suggestive evidence that these macromolecules are glycoproteins. It was significant that these same proteins were major components of soluble antigen since Frygin (4) has demonstrated the presence of glycoprotein in soluble antigen obtained from R. prowazekii. Other studies (see paragraph II) concerned with a serologically reactive fraction derived from soluble antigen also indicated a constant association of protein and carbohydrate after various separative procedures, again suggesting the presence of glycoproteins. The protein composition of typhus soluble antigen included proteins 1, 3, 4, 5, and 6. Thus, soluble antigen was comprised of all major cell envelope proteins except protein 2. We did not examine soluble antigen from R. conorii since a spotted fever antigen had been carefully examined by Tzianabos et al (2). It is important to note, however, that R. rickettsii soluble antigen analyzed by them on polyacrylamide gels was also devoid of a major protein (protein 2 in the nomenclature of this laboratory). It is possible that protein 2 may contribute to the species-specific complement-fixation antigen that is prepared by extensive washing of rickettsiae to remove soluble antigen. The identification of protein 4 as a peripheral protein in the cell envelope of R. prowazekii, its preliminary characterization as a glycoprotein and its contribution to typhus soluble antigen suggest that further study is warranted to determine its role in rickettsiae-host interactions.

II. Rickettsial indirect hemagglutination test: isolation of erythrocyte-sensitizing substance.

The indirect hemagglutination (IHA) test for human antibodies to typhus and spotted fever group rickettsiae has received renewed attention because of favorable results obtained in rickettsial

diagnostic studies comparing this technique with other serological procedures such as the complement-fixation, microagglutination, immunofluorescence and Weil-Felix tests (5). The IHA procedure is technically uncomplicated and practicable for routine diagnostic use, particularly when glutaraldehyde-stabilized erythrocytes are used as a carrier for rickettsial antigen (5). However, the nature of this antigen, termed erythrocyte-sensitizing substance (ESS), remains obscure. Originally, the preparative technique for ESS employed a preliminary ether extraction of infected yolk sacs to partially purify the rickettsiae, followed by treatment with hot alkali to produce an antigenically reactive substance that would adhere to red blood cells (6). More recently, ESS has been prepared from rickettsiae propagated in tissue culture and subsequently purified by sucrose gradient centrifugation. The source of ESS has been localized in the cell envelope fraction of rickettsiae, but its relationship to other serological reagents believed to be derived from the rickettsial cell envelope is obscure. The purposes of this investigation were to: 1) identify and quantify ESS-precursor and ESS in rickettsial extracts; 2) determine the physical and antigenic relationship between ESS and rickettsial complement-fixation antigens and; 3) determine the chemical nature of serologically reactive components of ESS.

A. Extraction of ESS-precursor with diethyl ether.

Rickettsial suspensions prepared as aqueous dilutions of infected yolk sacs, can be partially clarified by treatment with diethyl ether, which solubilizes some yolk sac lipids in the organic phase. After phase separation, the aqueous layer is known to contain particulate rickettsiae, a soluble group-specific antigen reactive in the CF test, and a precursor substance which yields ESS after treatment with hot alkali. It was of interest to determine if ESS-precursor remained bound to rickettsiae during ether extraction or was released in a soluble form. Rickettsiae were subjected to either short multiple extractions or a lengthy single extraction with diethyl ether at 26C. The aqueous phase was separated into particulate and soluble components by centrifugation at 12,000 x g for 60 min at 4C. Each component was treated with hot alkali, dialyzed, and assayed for ESS content by a modified IHA technique. Table 1 shows that approximately 50% of the ESS-precursor was extracted from R. prowazekii after 5 min exposure to ether. Neither single, long-term exposure to the solvent, nor multiple, short-term extractions achieved complete solubilization. Therefore, 5 min extraction at 26C was used in all further studies for preparation of ESS-precursor.

Table 1. Comparison of ESS-precursor recovery after single or multiple extractions of typhus rickettsiae with diethyl ether.

Fraction	Length of extraction	
	5 min	24 h
Aqueous supernatant from first extraction	4,096[a]	4,352[a]
Aqueous supernatant from second extraction	1,760	--
Aqueous supernatant from third extraction	408	--
Corpuscular material remaining after extraction	928	4,096

[a] Fractions were treated with hot alkali, dialyzed, and assayed for ESS activity by the modified IHA technique. ESS quantification compensated for the total volume of the fraction.

B. Physical relationship of soluble CF antigen and ESS-precursor.

The soluble, aqueous phase material derived from diethyl ether extraction of infected yolk sacs was concentrated 10X by TCA precipitation, adjusted to pH 7.2, layered onto a potassium tartrate density gradient and centrifuged at 148,000 x g for 45 h at 4C. Figure 7 shows that soluble CF antigen sedimented as two discrete components, one banding sharply at fractions 8-10, and the other more broadly at fractions 25-37. No ESS activity was demonstrable in any untreated fraction of the gradient. Fractions were pooled as shown by the brackets in Fig. 7, dialyzed to remove the anticomplementary potassium tartrate, retested for CF activity, treated with hot alkali, dialyzed, and assayed for ESS by the modified IHA technique. Table 2 indicates the CF and modified IHA reactivity of these pools before and after treatment with NaOH. It is apparent that ESS was obtained only from those fractions of the gradient which exhibited CF activity. Particulate rickettsiae, pelleted from the aqueous phase after ether extraction, were also analyzed on a potassium tartrate gradient. A single band of CF antigen was

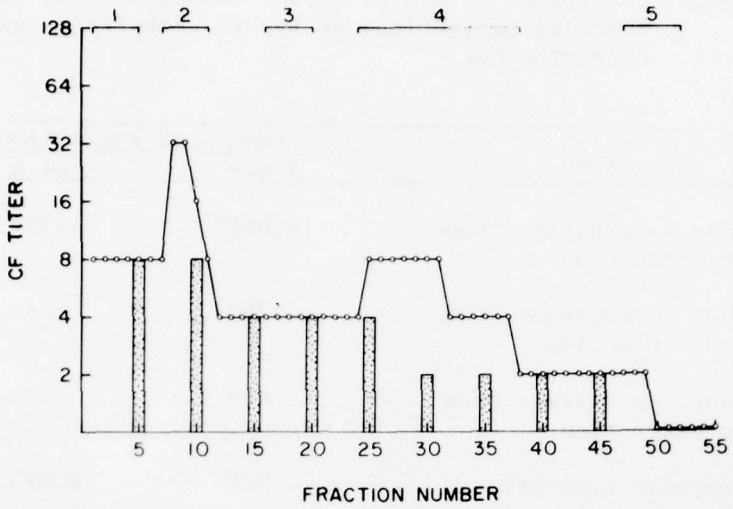


Fig. 7 Sedimentation of soluble, complement-fixing rickettsial antigen from R. prowazekii in a potassium tartrate density gradient o——o; complement-fixation titer ▨; anticomplementary activity. Brackets and associated numbers identify fractions which were pooled for subsequent analysis.

observed at fractions 8-12 (Fig. 8). This band was the only portion of the gradient which evidenced ESS activity after treatment with hot alkali (Table 2). Similar results were obtained when R. conorii was extracted with diethyl ether and the soluble and particulate aqueous phase antigens were centrifuged in potassium tartrate gradients. Although particulate rickettsiae and the denser components of soluble antigen banded in a similar region of the potassium tartrate gradient after equilibrium centrifugation, there were clear differences in their physical properties. The particulate, ether-extracted rickettsiae sedimented in a sucrose velocity gradient, while the soluble antigen did not enter a similar sucrose gradient and all detectable CF activity remained at the meniscus. The particulate and two soluble CF antigens possessed physical properties that enabled them to be separated by either density or velocity gradient centrifugation. In order to determine if the ESS derived from these CF antigens was similarly distinct, samples were centrifuged to equilibrium in potassium tartrate gradients.

Table 2. Recovery of CF and ESS activity after equilibrium centrifugation of soluble and particulate typhus CF antigen.

Pool	Before alkali treatment		After alkali treatment	
	CF	ESS	CF	ESS
Soluble antigen				
1	< 2[a]	< 2	< 2	< 2
2	640	< 2	320	2,560
3	< 2	< 2	< 2	< 2
4	896	< 2	< 2	1,792
5	< 2	< 2	< 2	< 2
Particulate antigen				
1	< 2	< 2	< 2	< 2
2	640	< 2	320	2,560
3	< 2	< 2	< 2	< 2
4	< 2	< 2	< 2	< 2
5	< 2	< 2	< 2	< 2

[a] Pools were assayed for ESS activity by the modified IHA technique. ESS and CF quantification compensated for the total volume of the pool.

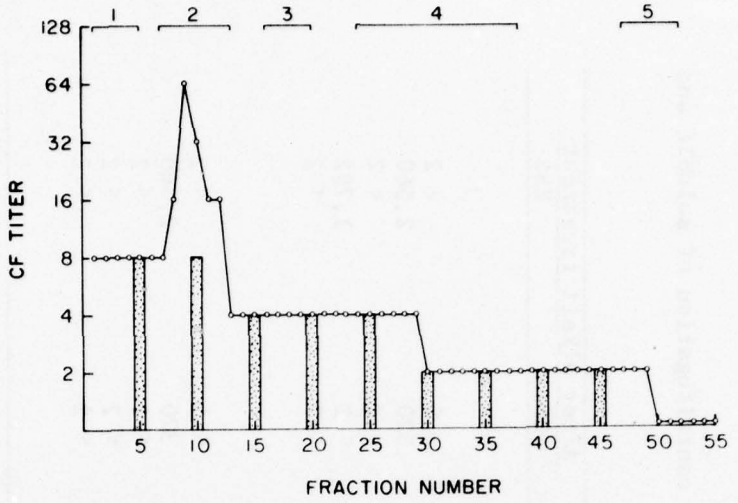


Fig. 8 Sedimentation of ether-extracted, particulate, complement-fixing rickettsial antigen from R. prowazekii in a potassium tartrate density gradient. o——o complement-fixation titer; [:] anticomplementary activity. Brackets and associated numbers identify fractions which were pooled for subsequent analysis.

Figure 9 demonstrates that ESS derived from particulate CF antigen, each soluble CF antigen and crude aqueous phase rickettsiae all sedimented chiefly as a broad, diffuse band in the center of the gradient. This sedimentation profile was observed with ESS derived from the CF antigen of both R. prowazekii and R. conorii. A small peak of ESS activity at fractions 5-7 was routinely observed after alkali treatment of ether-extracted particulate organisms. Based on the sedimentation profile of ether-extracted rickettsiae in potassium tartrate gradients (Fig. 8), this small peak of activity may represent particulate material from which ESS was incompletely solubilized by hot alkali. ESS purified by this gradient technique was susceptible to inactivation by potassium tartrate. A peak of ESS containing 353 units of serological activity, as assayed by the modified IHA test, became completely unreactive after 4 h at 26C or 24 h at 4C. The loss of reactivity was not observed when crude ESS was mixed with a similar concentration of potassium tartrate. Purified ESS could be protected from inactivation by the addition of bovine plasma albumin at a final concentration of 4 mg/ml.

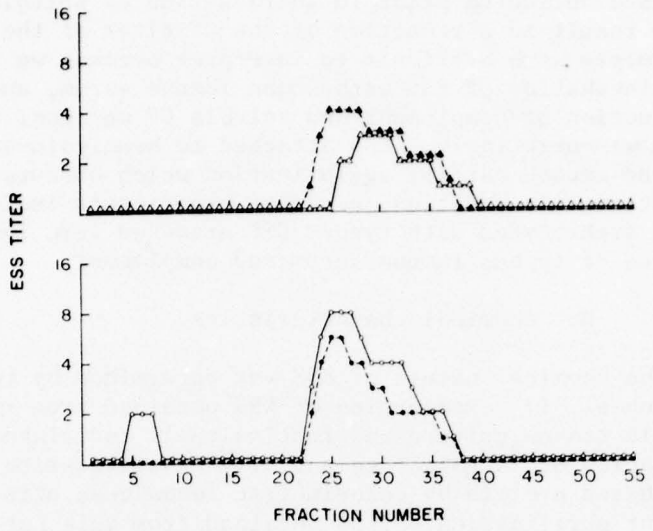


Fig. 9 Sedimentation in potassium tartrate density gradients of ESS derived from various complement-fixing antigens of R. prowazekii. Δ——Δ soluble CF antigen pool 2; ▲----▲ soluble CF antigen pool 4; o——o ether-extracted particulate CF antigen; ●----● crude aqueous phase rickettsiae.

C. Antigenic relationship of soluble CF antigen and ESS.

It appears that ESS was derived from CF antigens, and that alkali treatment converted discrete CF components to substances which were indistinguishable from each other in a potassium tartrate gradient. We investigated the antigenic relationship between ESS and its precursor substances through use of an IHA inhibition test. A reference typhus antiserum with a titer of 4,096 in the IHA test was incubated with soluble typhus CF antigen for 1 h, then free antibody was quantified in the IHA test. The titer was reduced to 4, although a control preparation of spotted fever soluble CF antigen had no effect on the IHA titer. Five different lots of typhus soluble CF antigen were tested, with each showing similar results. We also attempted to use typhus ESS to inhibit the interaction of typhus soluble CF antigen and human antibody. We reasoned that if ESS was able to interact with antibodies directed against CF antigens,

but was unable to fix complement, then the addition of ESS to a reference antiserum prior to introduction of soluble antigen should result in a reduction of the CF titer of the serum. These experiments were difficult to interpret because we found that after incubation of ESS with human immune serum, and subsequent introduction of complement and soluble CF antigen, the indicator system was unreliable. ESS attached to hemolysin-sensitized srbc and caused partial agglutination which obscured the normal CF pattern. In addition, separate experiments indicated that normal erthrocytes with typhus ESS attached were lysed in the presence of typhus immune serum and complement.

D. Chemical characteristics.

The chemical nature of ESS was determined by two different approaches: i) examination of ESS obtained from rickettsiae grown in tissue culture and intrinsically radiolabeled with ^{14}C amino acids or ^{3}H galactose and, ii) quantification of carbohydrate and protein by colorimetric techniques after density gradient purification of ESS obtained from yolk sac propagated organisms. The radioactive profile shown in Fig. 10 illustrates the results of centrifuging, in the same potassium tartrate gradient, ESS obtained from rickettsiae radiolabeled with amino acids or galactose. Three peaks of radioactivity were observed, but as expected, only two peaks, banding at fractions 9-12 and 26-35, exhibited ESS activity. Rickettsial antigen sedimenting in these regions of the gradient incorporated both radiolabeled galactose and amino acids. It is also clear that the potassium tartrate gradient separated ESS from serologically unreactive material which banded at fractions 37-54. Similar results were achieved with ESS obtained from radiolabeled R. conorii. A chemical analysis of ESS by colorimetric techniques also indicated the presence of both carbohydrate and protein. In order to obtain sufficient material for chemical analysis, soluble antigen obtained by ether extraction of typhus rickettsiae was TCA precipitated. This step resulted in a 20-fold concentration of CF antigen, which was treated with hot alkali and centrifuged on multiple potassium tartrate gradients. The major peak of ESS activity, located at fractions 23-38 (see Fig. 9), was obtained from each gradient, pooled, dialyzed and lyophilized. When resolubilized, the final preparation of ESS was concentrated approximately 100-fold from the original ESS. This preparation contained 2.2 mg/ml of protein and 1.2 mg/ml of carbohydrate. The contribution of each of these moieties to serological reactivity was determined by treating samples of typhus ESS with trypsin or sodium metaperiodate. Table 3 shows that trypsin treatment did not affect either binding of ESS to red cells, or the subsequent agglutination of sensitized cells by immune serum.

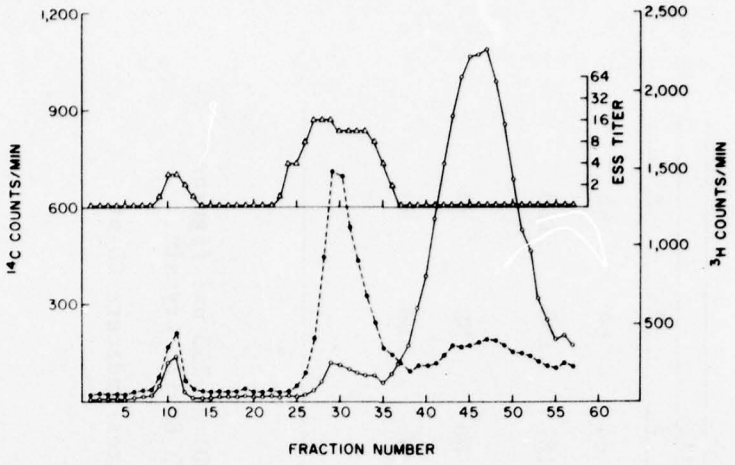


Fig. 10 Sedimentation of ESS extracted from radiolabeled R. prowazekii. o——o ^{14}C amino acids; ●----● ^{3}H galactose. The inset ordinate shows the ESS titer of radiolabeled fractions obtained from the potassium tartrate density gradient.

Periodate, on the other hand, totally ablated serological reactivity of ESS within 15 min. Identical data were obtained with spotted fever ESS. In order to separately assess the role of carbohydrate in ESS binding, we employed a preparation radiolabeled with ^{3}H galactose. Table 4 indicates that erythrocytes incubated with normal typhus ESS bound approximately 3.5X more radioactivity than those exposed to periodate-treated ESS. The quantity of serologically reactive ESS bound to erythrocytes, as measured by the modified IHA test, indicated a more severe reduction in biological activity than could be accounted for solely by the reduction in binding. The effect of periodate treatment on the capacity of ESS to interact with serum antibody was examined by use of an IHA inhibition test. An aliquot of typhus reference antiserum which exhibited a titer of 4096 in the IHA test was exposed to equal quantities of normal and periodate-treated typhus ESS for 1 h, then free antibody was quantified by the IHA procedure. Absorption of the serum with normal ESS reduced the antibody titer to 256, while serum exposed to periodate-treated ESS showed a titer of 1024.

Table 3. Effect of trypsin and sodium metaperiodate on typhus ESS.

Agent	Substrate	Time (h)					
		0	0.25	1	3	6	24
Enzite-trypsin (10 mg/ml)	ESS	64[a]	64	32	64	64	64
	BAEE	.186[b]	.182	.182	.187	.183	.188
$NaIO_4$ (0.05M)	ESS	64[a]	< 2	< 2	ND[d]	ND	ND
	α-methyl mannoside	.282[c]	.143	.103	ND	ND	ND

[a] ESS activity quantified by the modified IHA technique.

[b] Hydrolysis of BAEE measured as increase in optical density (OD) at 253 nm; figures indicate Δ OD at each time point after five min treatment with Enzite-trypsin.

[c] Oxidation by $NaIO_4$ measured as increase in OD at 225 nm; figures indicate OD at each time point as compared to a standard solution of $NaIO_4$.

[d] ND = not done.

Table 4. Effect of periodate treatment on the binding of typhus ESS to erythrocytes and their subsequent agglutination by immune serum.

^{3}H galactose labeled ESS	Radioactivity bound to srbc (cpm)	ESS titer
normal	424	128
periodate-treated	125	< 2

Diethyl ether extraction of rickettsiae has been employed routinely to partially remove contaminating host cell lipids from organisms in initial studies with ESS, but it was not clear whether the solvent, which is known to extract CF antigen, also liberated ESS-precursor from particulate rickettsiae. Our studies indicated that ESS-precursor, as well as soluble CF antigen, was rapidly extracted from rickettsial organisms by diethyl ether, and that soluble CF antigens from both typhus and spotted fever organisms could be resolved into two serologically reactive fractions by centrifugation in potassium tartrate gradients. The particulate rickettsiae remaining after brief ether extraction sedimented in the same region of the gradient as the denser form of soluble antigen. Other workers (2) have reported that particulate spotted fever rickettsiae were separable from soluble components during sedimentation in potassium tartrate-glycerol gradients, but only after lengthy extraction of formalized organisms with ether. In any case, our work indicates that the particulate CF antigen is distinct from the denser form of soluble CF antigen, since the 2 species are separable on rate-zonal sucrose gradients, suggesting a substantial difference in physical conformation. Our gradient centrifugation procedures were initiated with the goal of separating ESS-precursor from CF antigen. However, constant association of ESS-precursor activity with CF activity, and the failure to identify ESS-precursor in any gradient fraction which did not also possess CF activity, soon suggested to us that the immediate precursor of ESS was CF antigen. It appeared that alkali treatment modified the CF antigen in a manner which increased its capacity to bind to erythrocytes. Analysis of gradient-purified CF antigen before and after alkali treatment supported this hypothesis and further

indicated that achievement of erythrocyte binding properties was accompanied by loss of reactivity in the CF test. However, it is important to realize that although CF antigen is modified by the alkali treatment which produces ESS, distinct group-specific serological reactivity is retained (5, 6). We do not yet understand the effect of alkali on rickettsial CF antigen, but studies with other bacterial antigens also indicate that exposure to heat and high pH increases binding efficiency to red blood cells. Our studies indicated that such treatment of both species of soluble rickettsial CF antigen and particulate CF antigen resulted in similar products which met the criteria for ESS and were indistinguishable based on their sedimentation in potassium tartrate gradients. A small quantity of CF antigen associated with particulate rickettsiae was apparently converted to ESS by alkali without detachment from the organism. This finding was compatible with electron microscopic observations of Tzianabos et al. (2), who reported that cell wall particles analogous to soluble CF antigen remained attached to spotted fever organisms even after extensive extraction and purification. The antigenic relationship between soluble CF antigens and ESS was confirmed by the IHA inhibition test. Soluble CF antigen was capable of interacting with a reference antiserum and substantially reducing its reactivity in the IHA test. However, Chang et al.(6) suggested that antibodies reactive against particulate, washed, specific CF antigen were distinct from those reacting with ESS. These investigators also reported that ESS-antibody complexes were unable to fix complement in a CF test. Our results supported this observation but indicated that if ESS was bound to erythrocytes, then reacted with appropriate antibody, the complex would fix complement and result in lysis of the cells. Chemical analysis of typhus ESS obtained from potassium tartrate gradients indicated a protein to carbohydrate ratio of 2:1. Previous analysis of crude preparations (5) indicated a ratio of 17:1 for typhus group ESS. These data suggested that our isolation procedure removed a substantial quantity of protein, although such purification was accompanied by ESS instability. Centrifugation of crude radioactive ESS on potassium tartrate gradients also indicated that ESS was a carbohydrate-protein complex which complex sedimented as one major peak of serological activity separated from other radiolabeled material. The role of protein and carbohydrate components of ESS in the IHA test is undefined, but oxidation with periodate and enzymatic proteolysis both suggested an important function for the carbohydrate moiety. This finding was compatible with observations in other bacterial systems, but the role of carbohydrate seemed complex, since periodate oxidation affected binding of ESS to erythrocytes as well as interaction of ESS with serum antibody. Our studies do not preclude a requirement for protein in both of these activities, and more detailed investigations are required to fully define the

role of both ESS components. The usefulness of ESS may not be limited to its employment as a diagnostic reagent. ESS recovered from both typhus and spotted fever group rickettsiae possess common properties of erythrocyte binding and similar sedimentation in potassium tartrate gradients, yet retain discrete group-specific antigenic determinants capable of interacting with humoral antibody. It is possible that mild chemical or enzymatic degradation of ESS could selectively remove components conferring common biological or physical properties and result in a relatively simple substance that could be examined for antigenic determinants conferring group specificity. Previous studies from this laboratory (1) have examined intact rickettsial organisms from the typhus and spotted fever groups in a comparative manner using PAGE analysis. Although distinct differences were observed, protein patterns were complex, and a simple subcellular component such as ESS, which retains group-specificity could be valuable for more detailed analyses.

III. Development of an R. tsutsugamushi plaque reduction test.

Current serologic tests for studying the immune response to scrub typhus infection quantify circulating antibody, but only the toxin neutralization test provides information concerning the in vivo protective capacity of antibodies. This procedure is disadvantageous, however, because it requires concentrated suspensions of rickettsiae and the intravenous inoculation of mice. Also, the test applicability is restricted to only a few strains of R. tsutsugamushi. Recently, Kenyon and McManus (7) reported an antibody plaque reduction technique for the spotted fever group rickettsiae which could be useful in assessing the effect of immunoglobulins on rickettsia-host cell interactions. Their procedure consisted of reacting the rickettsiae with specific anti-rickettsial sera made in monkeys. Secondary antibody (anti-monkey immunoglobulins) was added after incubation, and the resultant mixtures reincubated and plaqued in chick embryo cell culture.

The purpose of the study described below was to develop a plaque reduction technique that might be useful in discriminating between strains of scrub typhus rickettsiae. Of primary importance in achieving this goal was our previous development of a plaque assay technique and preparation of triply plaque-purified stocks of 3 strains of R. tsutsugamushi (8). Preliminary experiments utilized R. tsutsugamushi, strain Gilliam, heat-inactivated anti-Gilliam serum prepared in BALB/c mice, and heat-inactivated rabbit-antimouse serum (secondary antibody). Results were very

inconsistent because addition of the secondary antibody elicited a precipitation reaction, which was dilution dependent. Primary antibody alone was also attempted. A small reduction in the number of plaques was evident at the lower dilutions of antibody in some cases; however, an apparent enhancement of plaque formation also occurred at low dilutions in many instances. We felt the enhancement could possibly be due to the protective effect of the serum as opposed to the brain heart infusion broth. In a second set of experiments the diluent was changed to non-immune serum, and the antiserum was prepared in New Zealand white rabbits by a single IV injection of approximately 5×10^7 plaque-forming units (PFU) of cell culture-propagated, plaque-purified Gilliam strain rickettsiae. The rabbits were bled on day 28, and the sera separated and filter sterilized. Serum from non-immune rabbits was treated in the same manner. Prior to use all sera were heat-inactivated at 56C for 30 minutes. Reaction of the Gilliam strain with 4-fold dilutions of its homologous antiserum resulted in a significant reduction (94%) in the number of plaques at the lowest antiserum dilution. Secondary antibody was not required to attain this value. Antisera against two other prototype scrub strains, Karp and Kato, were then prepared in rabbits in the same manner as the Gilliam antiserum. Each strain of R. tsutsugamushi was reacted with 4-fold dilutions of heat-inactivated homologous, heterologous, and non-immune sera, and then plaqued in irradiated L-929 cells. No secondary antibody reaction was used. The results are summarized in Table 5.

Table 5. Homologous and heterologous plaque reduction by prototype strains of Rickettsia tsutsugamushi.

Antigen	% PR by antiserum*		
	Karp	Gilliam	Kato
Karp	70	9	34
Gilliam	26	94	36
Kato	42	66	75

* per cent plaque reduction (% PR) = $100 - \frac{\text{treatment mean} \times 100}{\text{control mean}}$

Homologous sera plaque reductions ranged from 70% to 94% while the heterologous sera reductions ranged from 9% to 66%. The 66% plaque reduction occurred with Gilliam antisera and Kato strain organisms. These possible antigenic relationships have not been observed previously using immunofluorescence and complement fixation tests. These data suggest that the plaque reduction test may reveal new relationships between strains of scrub typhus rickettsiae that are not apparent by other tests.

IV. Antigenic characterization of plaque-purified scrub typhus rickettsiae.

Presently there are a large number of strains of Rickettsia tsutugamushi available and more are being isolated every year. Many of these isolates could conceivably be composed of multiple strains. To accurately characterize any of the strains, it becomes necessary to insure that they are "pure". Five strains isolated in Thailand in 1962 and 1963 were selected for plaque purification. These have previously been shown to exhibit little or no relationship to the 3 prototype strains, Karp, Kato, and Gilliam, according to complement fixation tests as reported by Elisberg et al. (9). These strains are designated TA 763, TA 716, TA 678, TA 686, and TH 1817. All except TH 1817 (a human isolate) were isolated from rodents. The purpose of this project is to perform an antigenic comparison of plaque-purified stocks of the 5 Thai strains and the 3 original prototype strains. The comparison will be made on the basis of the results from plaque reduction tests as well as those from complement fixation, immunofluorescence, mouse virulence, and cross-neutralization tests. This project is in the initial stage, the plaque purification of the 5 Thai strains. All have been shown to plaque satisfactorily in monolayers of irradiated L-929 cells and have been subjected to at least one cycle of the triple plaque purification scheme.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 096 Biochemical and Immunological Characterization of Rickettsial Proteins

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Presentations:

1. Eisemann, C.S. and Osterman, J.V. Analysis of rickettsial erythrocyte sensitizing substance. Annual Meeting of the American Society for Microbiology. Las Vegas, Nevada, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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77 10 01	D. Change	U	U	NA	NI	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	097
b. CONTRIBUTING				
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(U) Antigenic Characterization of Sandfly Fever Viruses

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23 (U) To define the antigenicity and immunogenicity of the proteins of Sandfly Fever viruses, to develop rapid means of specific diagnosis, to study the immune response to infection and to develop means for reducing disability. Sandfly fever affected both American and German troops during World War II. Antigenically distinct strains of these viruses exist in the Mediterranean Basin, Central America and Asia.

24 (U) Contemporary virological and immunological methods are used to identify and compare the antigens of distinct strains of Sandfly Fever viruses. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 77 10 - 78 09 Sandfly (Phlebotomus) Fever viruses were selected on the basis of their association with human disease and include Naples, Sicilian, Candiru, Chagres, Punta Toro, Itaporanga and Karimabad. Cloning of these viruses by plaque purification was hampered by the observation that some of them (Naples and Sicilian) would not produce plaques under a standard agar overlay. Dimethyl sulfoxide in combination with diethylaminoethyl dextran was found to facilitate plaque formation by both viruses. Naples virus retained its dependency on the chemicals for plaque formation after cloning and passage from mouse brain into Vero cells, but the Sicilian virus did not. Hyperimmune mouse ascitic fluids have been prepared to uncloned viruses and tested by plaque reduction neutralization tests against cloned viruses. Characterization of the cloned virus particles is in progress. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 77 to 30 Sep 78.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 097, Antigenic Characterization of Sandfly Fever Viruses

Investigators:

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Description

To define the characteristics and antigenicity of the proteins of Sandfly Fever Viruses, to develop improved means of specific identification, to study the immune response to infection and to develop means of reducing disability. Emphasis is placed on Sandfly Fever Viruses that are capable of infecting humans.

I. Dimethyl Sulfoxide Enhancement of Phlebotomus Fever Virus Plaque Formation.

A. Background

Phlebotomus Fever viruses are grouped primarily on an epidemiological and partial serological basis and represent a collection of some 23 agents isolated from humans, sandflies and animals (Tesh et al., 1975, 1976). Members of the group do not cross react with one another as extensively by the complement fixation test as do the Bunyaviruses, but they are considered Bunyavirus-like because of morphological similarities (Murphy et al., 1973). The relationships of Phlebotomus Fever viruses to one another and to the Bunyaviruses could be approached through genetic studies (Gentsch et al., 1977); however, such studies utilized plaque formation under a rigid agar overlay for cloning and purification procedures, as well as isolating potential recombinants. A method currently used employs gum tragacanth which is a semisolid to liquid substance that does not restrict virus particles to the proximity of the plaque. The Naples and Sicilian viruses do not form plaques under rigid agar at virus concentrations 10 times greater than that required to kill suckling mice. In

this report we show that dimethyl sulfoxide enables plaque formation by Naples and Sicilian virus whereas it has little, if any, effect on other Phlebotomus Fever group viruses or representatives of the alphaviruses (Sindbis) and flaviviruses (dengue).

B. Methods

1. Viruses and cell cultutes. The following Phlebotomus Fever group viruses were obtained as either infected Vero cell, suckling mouse brain (SMB), or suckling hamster brain (SHB) from R.B. Tesh (NIAID, Honolulu) and R.E. Shope (Yale, New Haven): Aguacate (SHB 5), Cacao (Vero 4), Caimito (SMB 1), Candiru (SMB 11), Chagres (SMB 15), Chilibre (Vero 4), Frijoles (SHB 1), Icoaraci (SMB 2), Itaporanga (SMB 16), Karimabad (SMB 19), Naples (SMB 48), Nique (SMB 1), Punta Toro (SMB 12), and Sicilian (SMB 33). The viruses were propagated in suckling mouse brain for 2 serial passages to prepare hyperimmune mouse ascitic fluid (Brandt et al., 1967; Chiewsilp and Mc Cown, 1972). After the plaque assay was developed with Vero cells, the viruses were cloned by selecting and passaging isolated plaques for 3 serial passages. Additional passages were made in Vero cells to prepare seed virus for plaque reduction neutralization test (Russell et al., 1967). Sindbis (AR 339) and dengue-2 (New Guinea C viruses, SMB 10 and SMB 36, respectively) were used as representative Togaviruses.

The Vero cells were obtained from R.B. Tesh and propagated and maintained in 32 oz glass prescription bottles or 25 cm^2 plastic flasks (Falcon, Oxnard, Calif) using Minimal Essential Medium with Earle's salts (EMEM) containing 5 percent fetal bovine serum (heated 60°C x 30 min), 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 ug/ml).

2. Infection of cell cultures. a) Plaque assay: Virus dilutions were prepared in medium 199 containing 20 percent fetal bovine serum; 0.2 ml aliquots were placed on drained monolayers of Vero cells in 25 cm^2 flasks which were then incubated at 35°C for 90 min (adsorption period). Seven ml of a 1% agar overlay at 44°C was added and the flasks were incubated at 35°C for 7 days. The agar overlay consisted of (per 100 ml): 1 g Agar Purified (Difco, Detroit) and 72 ml double distilled water sterilized in the autoclave; the nutrient portion, after mixing with cooled agar, consisted of 10% 10 x concentrated medium 199 without phenol red and $NaHCO_3$, 0.02%

diethylaminoethyl-dextran, 0.5% each of 100 x concentrated essential vitamin mixture and 100 x concentrated essential amino acid mixture for Basal Medium Eagle (Microbiological Associates) and antibiotics. The indicated concentration of DMSO (Sigma Chemical Co.) was added to the standard overlay. The neutral red vital strain (added 7 days later) consisted of a 1:7500 dilution in 1 percent agar in normal saline (4 ml volume). The flasks were incubated for 4 hours at 35°C and overnight at room temperature before the plaques were counted.

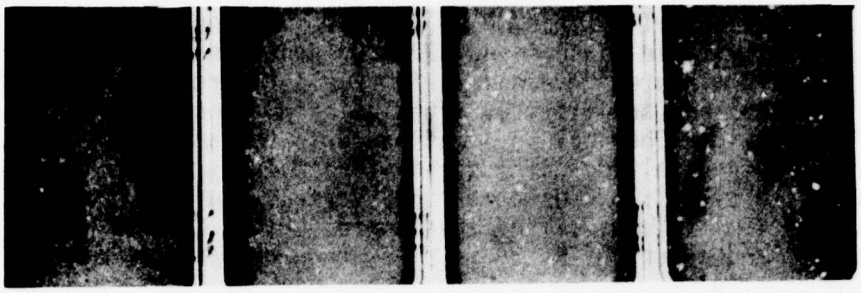
b) Growth curves: Confluent monolayers of Vero cells in 25 cm^2 flasks were infected at multiplicities of approximately 3. The inoculum was removed by rinsing the flasks 4 times with medium. Six ml of medium with or without the indicated concentrations of DMSO was added to the flasks and 1 ml was removed immediately (0 time sample). Additional 1 ml aliquot were removed at daily intervals, the volume being replaced with fresh medium. All samples were stored at -70° until assayed for infectivity.

C. Results

1. Naples virus replication and plaque formation with DMSO.

Most of the following studies were carried out with the Naples virus since it is one of the earliest Phlebotomus Fever group human pathogens. The mouse brain seed that contained 3.2×10^7 suckling mouse LD_{50}/ml contained only 2.5×10^6 pfu/ml by our plaque assay used for Togaviruses. Plaques were barely visible (less than 0.5 mm), and many assays yielded no visible plaques at all.

The effect of DMSO on plaque formation was tested by infecting Vero cells with a virus dilution containing 100-200 suckling mouse LD_{50} combined with the indicated concentrations of DMSO and overlaying the monolayers with agar containing the same concentrations of DMSO. Plaques which were barely visible under agar without DMSO became clearer and more numerous as the concentration of DMSO was increased from 0.1 to 1.0 percent (Fig. 1); the size of the plaques ranged from 0.5 to 1.5 mm. The plaques decreased in size and number with 2.5 percent DMSO.



0% 0.1% 0.5% 1.0%

FIGURE 1. Effect of several dimethyl sulfoxide concentrations on plaque formation by Naples Phlebotomus fever virus.

The effect of DMSO on the quantity of virus released by infected cells cultured in liquid medium was tested in the same manner; increasing concentrations of DMSO were included in the inoculum as well as the medium. Slightly more virus (up to 2.5 fold at 3 days) was released into the culture medium containing DMSO (Fig. 2). The quantity of virus released by infected cells cultured in 2.5 percent DMSO was similar to that shown for 0.1% DMSO in Figure 2.

The effect of DMSO on the infecting inoculum was examined by mixing the inoculum with an equal volume of normal medium and medium containing 2 percent DMSO. The final concentration of 1 percent DMSO was judged as the optimum concentration for plaque clarity and size. Replicate flasks received an agar overlay with and without 1 percent DMSO after the adsorption period. No difference in plaque titer was observed between the inoculum with and without DMSO when both sets of monolayers were overlaid with DMSO agar. The agar overlay without DMSO did not allow production of any plaques, even on monolayers infected with the virus-DMSO mixture (data not shown).

2. Effect of heparin and DEAE-dextran in combination with DMSO.

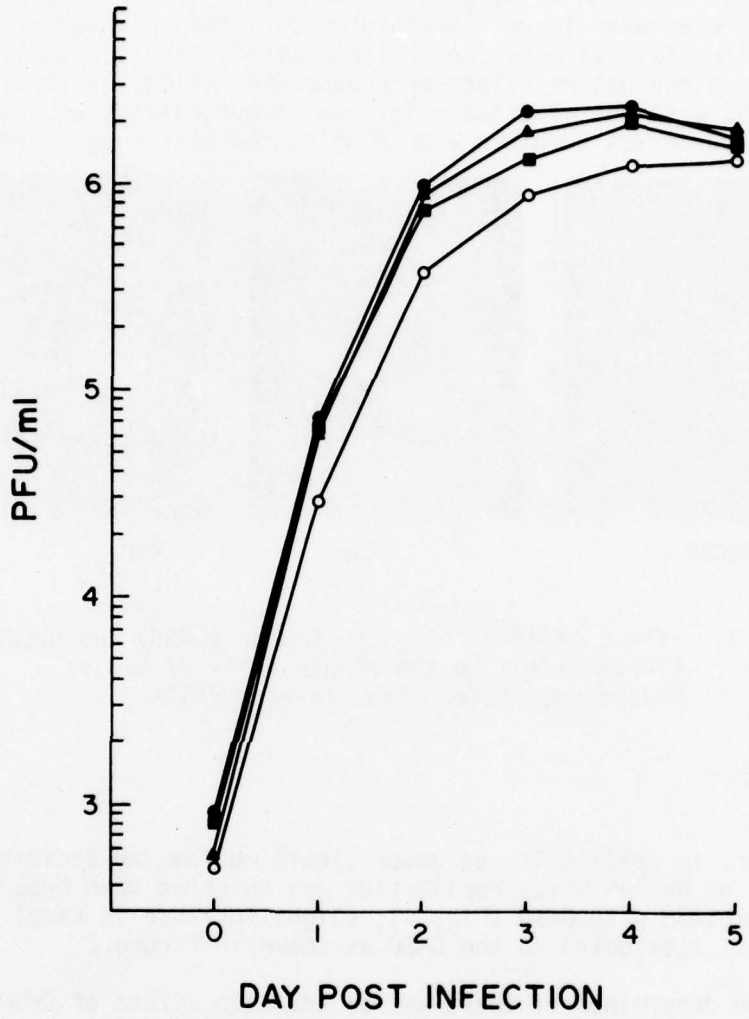
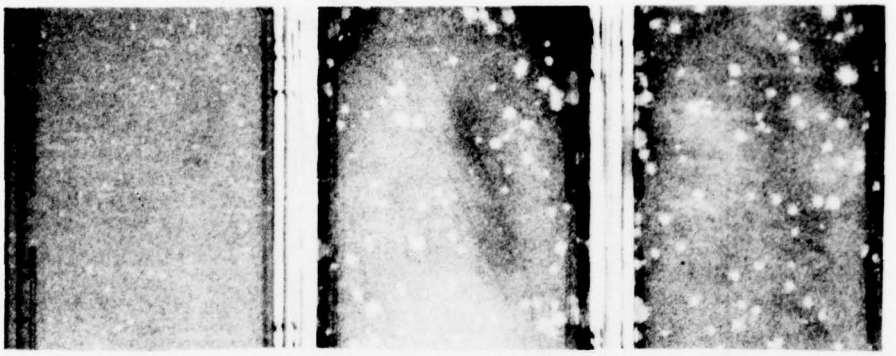


FIGURE 2. Replication of Naples sandfly fever virus in Vero cells cultured under several concentrations of dimethyl sulfoxide. Symbols: O, No DMSO; ■, 0.1% DMSO; ▲ 0.5% DMSO; ● 1.0% DMSO. The multiplicity of infection was 2.7.

The inclusion of heparin (10 units/ml) in the agar overlay has been used in our laboratory to clarify plaques produced by various flaviviruses (unpublished ovservations). While heparin alone has no effect on enhancement of Naples virus plaques, a slight additive effect on plaque clarity was observed when heparin was combined with DMSO in the agar overlay (Fig. 3).



STANDARD DMSO DMSO + HEPARIN

FIGURE 3. Effect of 1% dimethyl sulfoxide (DMSO) and heparin (10 units/ml) on the plaque assay of Naples Phlebotomus fever virus in Vero cells.

However, in cells cultured under liquid medium, no additive effect on Naples virus replication was observed when heparin was combined with DMSO (Fig. 4); slight increase in viral yield is attributed to the DMSO as shown in Figure 2.

We determined if there was an additive effect of DMSO on the DEAE-dextran component of the agar overlay. In a typical experiment, no plaques were visible on the monolayers infected with an estimated 100 pfu when DEAE-dextran was left out of the agar overlay containing DMSO. Infected monolayers under agar containing both DMSO and DEAE-dextran produced an average of 105 clearly visible plaques, and those monolayers under agar with DEAE-dextran but no DMSO contained an average of 18 barely visible plaques.

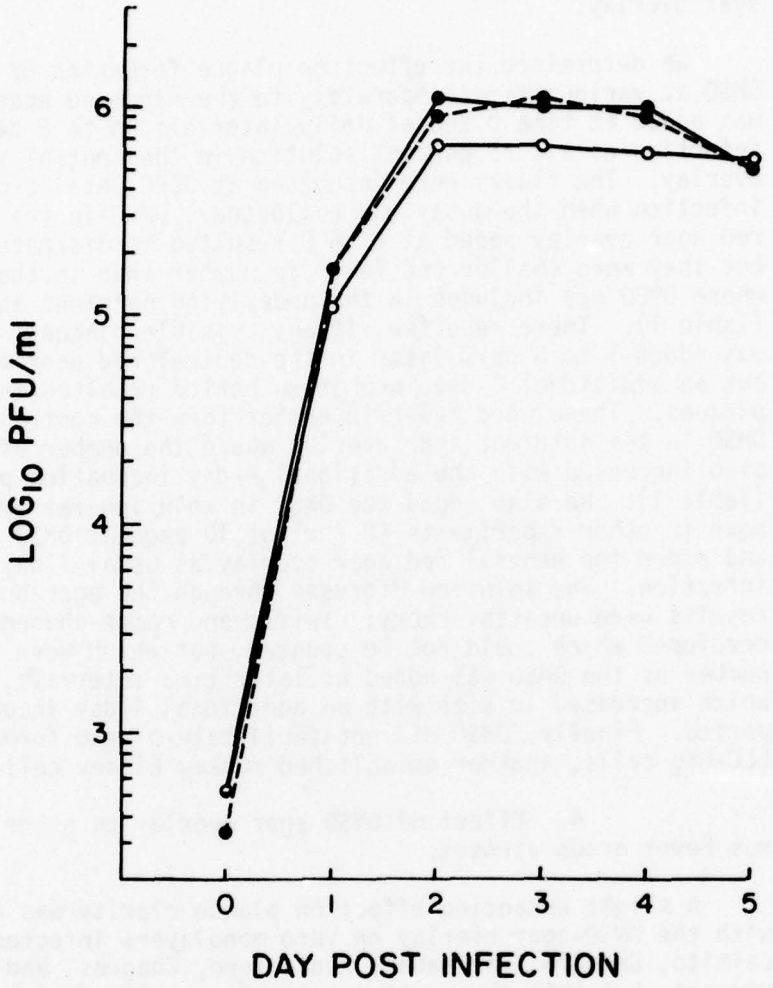


FIGURE 4. Effect of DMSO (1%) and heparin (10 units/ml) on the replication of Naples sandfly fever virus in Vero cells. Symbols: O, No DMSO or heparin; ● (broken line), DMSO + heparin; ● (solid line), DMSO alone. The multiplicity of infection was 3.2.

3. Effect of delayed addition of DMSO to the agar overlay.

We determined the effect on plaque formation of adding DMSO at various times separately to the hardened agar. DMSO was added at time 0 and at daily intervals up to 5 days after infection as a 2.75 percent solution in the neutral red agar overlay. The flasks were incubated at 35°C until 8 days post infection when the assay was evaluated. DMSO in the neutral red agar overlay added at time 0 resulted in discrete plaques but they were smaller and fewer in number than in the controls where DMSO was included in the underlying nutrient agar overlay (Table 1). There were few, if any, visible plaques when DMSO was added 1 to 5 days later in the neutral red agar overlay, but an additional 4-day incubation period resulted in visible plaques. These were fewer in number than the controls receiving DMSO in the nutrient agar overlay where the number of plaques also increased with the additional 4-day incubation period (Table 1). We also added the DMSO in solution rather than agar in other experiments (0.7 ml of 10 percent DMSO in medium) and added the neutral red agar overlay as usual 7 days post infection. The solution diffused through the agar but the results were unsatisfactory: swirls and comet-shaped streaks developed which could not be counted, but which were fewer in number as the DMSO was added at later time intervals, and which increased in size with an additional 4-day incubation period. Finally, DMSO did not facilitate plaque formation in LLC-MK_2 cells, another established monkey kidney cell line.

4. Effect of DMSO agar overlay on other Phlebotomus Fever group viruses.

A slight enhancing effect on plaque clarity was observed with the DMSO-agar overlay on Vero monolayers infected with Caimito, Candiru, Karimabad, Punta Toro, Chagres, and Chilibre viruses, but this observation was not associated with a significant increase in the number of plaques as it was with Naples and Sicilian viruses, (Table 2). There was no difference in plaque clarity or number with or without DMSO in the agar when Aguacate, Cacao, Frijoles, Icoaraci, Itaporanga, and Nique viruses were assayed. In general, the size of the plaques produced by these other viruses ranged from 3.0 to 5.0 mm as compared to plaques produced by the Naples and Sicilian viruses which ranged from 0.5 to 1.5 mm. The numbers of plaques at various dilutions of the viruses were consistent with the 10-fold dilution schedule.

Table 1. Effect of delayed addition of dimethyl sulfoxide to the hardened agar overlay*

Time of addition of DMSO	Number of Plaques	
	Day 8	Day 12
0 TIME	63,38	98,59
Day 1	0,0	73,54
Day 2	1,0	42,55
Day 3	1,2	59,44
Day 4	3,0	64,53
Day 5	3,4	69,61
Control (1% DMSO in first nutrient agar overlay)	88,80	136,125

*DMSO was added in a second agar overlay containing neutral red and 2.75 percent DMSO in 1 percent agar. All monolayers were infected with an estimated 100 PFU calculated from a previous titration.

Table 2. Effect of dimethyl sulfoxide (DMSO) on Phlebotomus group virus plaque assays in Vero cells

Virus	Source	Agar Overlay		Ratio
		Standard	DMSO	DMSO/Standard
Naples	mouse brain (a)	2.5×10^6*	2.4×10^7	9.6
	(b)	2.2×10^6*	2.2×10^7	10.0
	Vero	6.3×10^5*	4.8×10^6	7.6
Sicilian	mouse brain	9.0×10^5*	1.1×10^7	12.2
	Vero	5.4×10^6	4.8×10^6	0.9
Karimabad	mouse brain	1.0×10^8	1.6×10^8	1.6
	Vero	2.6×10^7	3.7×10^7	1.4
Punta Toro	Vero	7.4×10^7	7.5×10^7	1.0
Chagres	Vero	2.0×10^6	2.7×10^6	1.3
Candiru	Vero	4.0×10^6	6.4×10^6	1.6
Itaporanga	Vero	3.7×10^7	4.2×10^7	1.1
Icoaraci	Vero	9.2×10^6	9.1×10^6	1.0
Cacao	Vero	5.4×10^6	5.1×10^6	0.9
Chilibre	Vero	3.7×10^6	3.9×10^6	1.0
Caimito	Vero	3.5×10^6	3.6×10^6	1.0
Frijoles	Vero	2.8×10^7	4.2×10^7	1.5
Nique	Vero	1.6×10^7	2.1×10^7	1.3
Aguacate	Vero	2.2×10^8	1.9×10^3	0.0

*Difficult to observe and count.

5. Virus neutralization tests assayed under DMSO agar.

Homologous and heterologous plaque reduction neutralization tests were carried out with some of the Phlebotomus group viruses in a standard manner except that 1 percent DMSO was included in the agar overlay applied after the adsorption period. The results (Table 3) were similar to those reported previously (Tesh et al., 1976) using a gum tragacanth overlay to measure unneutralized virus; e.g. when comparing Naples and Sicilian reagents, the Sicilian HMAF was the more potent antibody preparation and both antibody preparations were type specific.

6. Effect of DMSO agar overlay on Togaviruses. The plaque formation of Sindbis and Dengue-2 viruses were studied under our standard agar and DMSO agar. Sindbis plaques were somewhat larger but without an increase in number, while Dengue 2 plaques were identical in size and number under both kinds of agar overlay.

D. Discussion

Dimethyl sulfoxide (DMSO) included in an agar overlay on infected Vero cells enhanced plaque size and clarity of Naples and Sicilian Phlebotomus Fever viruses and increased the number of plaques as much as 10-fold. This pronounced effect of DMSO was not observed with other Phlebotomus Fever group viruses which produced very large plaques (3 to 5 mm) as compared to the Naples and Sicilian strains (0.5 to 1.5 mm). These observations represent a pronounced biological difference among the members of this group.

The effect of DMSO on Naples virus plaque size was clearly dependent on concentration, with 1 percent considered optimum. In a previous report (Cleaver, 1974), 1 percent DMSO was "near lethal" for CV1 cells, another African green monkey kidney cell line. It was proposed that near lethal concentrations of DMSO had a preferential deleterious effect on infected cells and that SV40 virus plaques were formed faster in the presence of 0.5% DMSO because of earlier cell lysis. In our studies, the Vero cells remained viable in the presence of 1% DMSO for up to 12 days. Generally, concentrations of 5% or higher were required to inhibit enzyme systems (Rammler, 1967). DMSO probably affects cell permeability by increasing the rate of movement of molecules in cell membranes (Jacob et al., 1964; Franz and Van Bruggen, 1967). Infected

Table 3. Plaque reduction neutralization titers of hyperimmune mouse ascitic fluids to the Phlebotomus fever group viruses assayed under DMSO agar.

Hyperimmune ascitic fluid	Virus										
	Naples	Sicilian*		Candiru	Caimito	Nique	Frijoles	Punta Toro	Chagres	Karimabad	Itaporanga
		DMSO	Standard								
Naples	2600[+]	0	0	0	0	0	0	0	0	0	10
Sicilian	0	4800	3100	0	0	0	30	20	0	0	10
Candiru	0	0	0	1000	0	0	0	40	0	0	0
Caimito	0	0	0	0	2560	0	0	0	0	0	0
Nique	0	0	0	0	0	80	0	15	0	0	0
Frijoles	0	11	12	0	0	0	640	0	0	0	0
Punta Toro	0	12	10	30	0	15	20	960	0	12	80
Chagres	0	15	13	0	0	0	0	0	1280	0	22
Karimabad	10	0	10	10	0	0	0	40	0	1280	80
Itaporanga	0	0	0	0	0	0	0	0	0	0	320

*The Sicilian neutralization test was assayed simultaneously under DMSO and standard agar.
[+]Reciprocal of highest dilution of ascitic fluid neutralizing 50% of the viral plaques.

cells stimulated in such a manner may have lost their viability sooner under the stress of viral replication at the expense of cellular processes required to respond to the cell surface stimulation of DMSO. The end result would be visible plaques because of earlier cell lysis as proposed previously, but probably not because of near lethal DMSO concentrations for normal Vero cells. The slight increase in yield of infectious virus from cells cultured in liquid medium with DMSO might be associated with earlier cell lysis but we did not observe earlier cytopathic effects.

DMSO was not effective in allowing plaque formation by Naples virus under agar unless DEAE-dextran was present. DEAE-dextran binds sulfated polysaccharides found in agar which inhibit the infectivity of certain viruses (Schulze and Schlesinger, 1963; Schulze, 1964). Thus,agar inhibitors of viral infectivity might have had to be removed with DEAE-dextran before the DMSO could enhance plaque formation. When we added DMSO at later daily time intervals to the hardened agar containing DEAE-dextran, an additional incubation period past the usual 7-day observation time was required for plaques to form. Either the infected cells required exposure to the DMSO for a certain period of time, or, there may have been a synergistic effect of DMSO and DEAE-dextran on agar inhibitors as well as the cells to allow plaque formation to occur.

DMSO was not required for adsorption or penetration since infection and growth of Naples virus occurred in cells cultured in liquid medium without DMSO. DMSO did not produce appreciable stability or disaggregation of potential virus clumps since titers of virus suspended in normal medium and DMSO medium were the same. There were other variables such as the cell culture substrate; a second monkey kidney cell line, LLC-MK_2 cells, would not support Naples or Sicilian virus plaques in the presence of DMSO and DEAE-dextran. Another variable was the passage history of the virus: both Naples and Sicilian viruses were passaged 5 times in Vero cells. However, the Vero passaged Sicilian virus did not require DMSO to form plaques as did the mouse brain passaged virus. Naples virus, on the other hand, still required DMSO to form plaques after Vero cell culture passage. The Vero cell propagated viruses were neutralized by antiserum to mouse brain propagated viruses indicating there was no change in the surface antigens of the passaged viruses.

Regardless of the mechanism of DMSO on plaque formation, the more prominent Phlebotomus Fever viruses can now be plaqued

under rigid agar overlay for cloning and genetic studies. Naples and Sicilian viruses could be plaqued previously only under gum tragacanth, a semi-solid to liquid substance which does not restrict the virus particles to the vicinity of the plaque. Using rigid agar, virus can be cloned from individual plaques which will allow easier characterization of subpopulations of the parent virus, some of which even may differ in their requirement for DMSO to form plaques.

Table 4. Characteristics of Cloned Phlebotomus Fever Virus Suspensions

Virus	Plaque Morphology (Size)	Titer (pfu/ml)
Karimabad		
KAR #1	large (4-5 mm)	1.1×10^8
KAR #2	mixed large & small	7.5×10^6
KAR #3	small (1-2 mm)	9.5×10^5
KAR #4	small (1-2 mm)	2.0×10^6
Chagres		
CHG #1	mixed large and medium	4.4×10^5
CHG #2	mixed large and small	6.5×10^7
CHG #3	medium (2-3 mm)	1.0×10^7
CHG #4	medium (2-3 mm)	7.0×10^7
Sandfly fever Sicilian		
SFS #1	mixed small and medium	5.0×10^7
Sandfly fever Naples		
SFN #1	small (1 mm)	1.3×10^7
Punta Toro		
PT #1	large (4-5 mm)	2.3×10^8
PT #2	medium (2-3 mm)	9.5×10^7
PT #3	medium (2-4 mm)	1.8×10^8
Itaporanga		
ITP #1	mixed small and medium	1.7×10^7
ITP #2	mixed small and medium	1.4×10^7
ITP #3	medium (2mm)	1.6×10^7
Candiru		
CDU #1	medium (2-3 mm)	1.6×10^7
CDU #2	medium (2-3 mm)	1.2×10^6
CDU #3	small (0.5-1.5 mm)	9.8×10^5
CDU #4	small (0.5-1.0 mm)	2.3×10^5

II. Evaluation of Phlebotomus Fever Group Virus Seeds

As a prerequisite to virus characterization, stock working virus seed preparations were made by plaque purification of seven Phlebotomus fever Group viruses selected for their recorded association with human disease: Karimabad (KAR), Chagres (CHG), Sandfly fever Sicilian (SFS), Sandfly fever Naples (SFN), Punta Toro (PT), Itaporanga (ITP), and Candiru (CDU) viruses. Each virus was plaque assayed on Vero cell monolayers using DMSO in the overlay medium and representative clones were then selected. At least four individual, well separated plaques, (representative of the plaque size distribution observed for each virus), were selected and an agar plug above the plaque was aspirated and diluted in growth medium. This virus containing material was further diluted and assayed on Vero cell monolayers: this cloning procedure was repeated three times. The agar plug obtained from the third "plaque pick" was used to infect a 32 oz. bottle of Vero cells and the supernatant fluids of this culture (harvested following the appearance of CPE) constituted the stock working seed virus. Details of these cloned virus seed suspensions are presented in Table 4.

The infectivity titers of the stock virus suspensions varied over a considerable range. Although it has not been determined that these are the maximum achievable titers, selected viruses were examined in a one-step growth curve (MOI = 1) in an attempt to estimate the titers to be expected following an additional pass in Vero cells. The growth curves for three representative viruses are presented in Figure 5. The maximum titers obtained at the plateau of the growth curve were very close to the titers of the cloned stock seed suspensions. It appeared that the separate virus seeds reflect the maximum yield of these viruses in Vero cells and, consequently, representative seeds for future use were selected on the basis of the maximum infectivity titer.

The effect of MOI on the replication of KAR #1 was studied in detail in an attempt to optimize conditions for maximum virus yield from Vero cells. Growth curves were performed at a variety of MOIs with samples taken through 115 hours. The essential points from these experiments are summarized in Table 5. Maximum titers (greater than 10^9 pfu/ml) were only obtained using an MOI of less than one virus per cell. Virus replication progressed more slowly at low MOIs as reflected by the titers obtained at 40 hours, but the final yield at the last time sample tested (115 hours) was higher at MOIs between 0.001 and 1.0. The highest titered stock seed suspensions of each of the other cloned

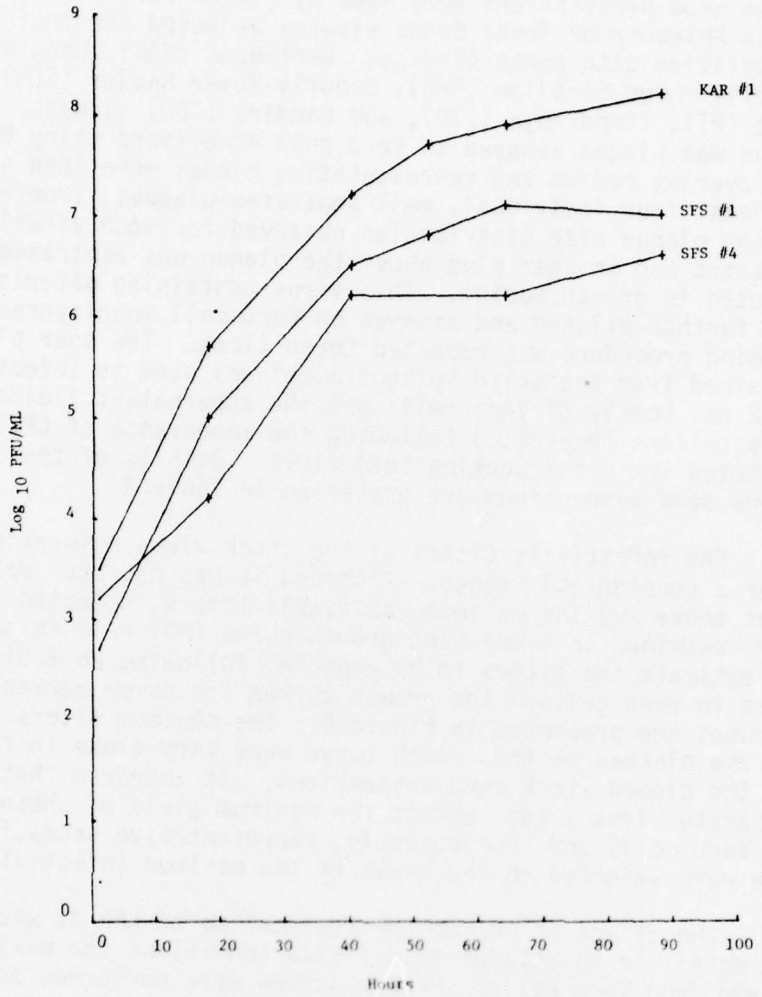


FIGURE 5. Representative Growth Curves of Phlebotomus Fever Viruses in Vero Cells - (MOI=1)

Table 5. Effect of MOI on the Replication of Karimabad Virus in Vero Cells

MOI	TITER AT 40 HRS (pfu/ml)	MAX. TITER (pfu/ml)	TIME OF MAX. TITER
55	5.2×10^7	2.3×10^8	67 hrs
5.0	1.5×10^7	1.0×10^8	115 hrs
1.0	8.5×10^6	4.0×10^8	115 hrs
0.5	1.1×10^7	2.5×10^9	115 hrs
0.1	5.0×10^6	2.6×10^9	115 hrs
0.01	1.8×10^5	2.4×10^9	115 hrs
0.001	1.0×10^5	5.0×10^8	115 hrs

Table 6. Effect of Actinomycin-D on Karimabad Virus Replication and Protein Synthesis in Vero Cells

Actinomycin-D (ug/ml)	Amino Acid Incorporation (% of control)	Virus Yield (% of control)
0 (control)	100	100
0.01	93	50
0.1	113	187
0.25	78	520
0.5	57/70	553/420
1.0	97/87	333/187
2.5	89/87*	193/167
5	88	100
10	74	167
20	89	133
40	93	93
100	44	30
Uninfected -0-	157	-

*Results from two separate experiments.

Phlebotomus fever viruses have also been tested in growth curve experiments using varying MOIs. Although maximum virus yields varied, the dependence on MOI appeared to parallel the data presented for KAR #1.

Two other cell lines in the laboratory have been investigated in growth curve experiments with each of the Phlebotomus fever viruses. The LLC-MK2 monkey kidney cell line consistently gave lower virus yields; however, BHK21 clone 15 baby hamster kidney cells gave virus titers comparable to Vero cells for some of these agents.

III. Preparation of radioactive viruses

As a prerequisite to virus radiolabeling experiments, the effect of the drug Actinomycin D was investigated with respect to its influence on KAR #1 virus yield and Vero cell protein synthesis. The results are summarized in Table 6. Vero cells were infected at an MOI of 0.4 (or mock infected in the case of the uninfected control), various concentrations of Actinomycin-D in deficient medium were added at 15 hours, a radioactive amino acid mixture was added at 20 hours and both supernatant and cells were harvested at 60 hours post-infection. Infectivity of the supernatant determined by plaque assay was used to calculate virus yield and acid precipitable counts in both the supernatant and solubilized cells in order to estimate the amount of incorporation of radioactive amino acids into protein. The Vero cells were not very sensitive to Actinomycin-D because there was only limited suppression of protein synthesis at other than very high drug concentrations; however, it did appear to increase virus yield over the range of 0.25 to 2.5 ug/ml. Unless better conditions for Actinomycin-D treatment yield greater suppression of host cell protein synthesis, the use of this drug for radiolabeling experiments remains questionable even though increases in virus yield were observed.

Many attempts to intrinsically radiolabel these viruses have not yet resulted in purified preparations with high specific activity. Some of the observations made relative to this problem may lead to procedures that will ultimately yield virus suspensions of high specific activity and will be presented here in summary form only. Most of the detailed information relates to KAR #1 virus.

The isotope ^{35}S-methionine is superior to a general label

^{3}H-amino acid mixture for radiolabeling these agents, probably because of the much higher specific activity of the ^{35}S reagent that is available. The use of methionine deficient medium has numerous disadvantages, not the least of which is a decreased yield of infectious virus and the release of a radiolabeled sedimentable particle which is smaller and more dense than infectious virus. The time of isotope addition to infected cultures appeared to be an important variable. Very little difference in radioactive virus yield was observed if ^{35}S-methionine was added at 6, 30 or 54 hours post-infection and virus harvested at 116 hours; however, significant increases in radioactive virus yields were observed when infected cultures were radiolabeled at both 6 and 54 hours post-infection.

IV. Characterization of structural proteins

Numerous methods for the concentration and purification of these viruses have also been examined. Precipitation with polyethylene glycol (PEG) 6000, appeared to be a superior means of concentration as opposed to direct pelleting by ultracentrifugation, which disrupted virion integrity. Obviously precipitation with PEG is not in and of itself a significant purification procedure since numerous radioactive proteins are present in this PEG pellet when examined by polyacrylamide gel electrophoresis (PAGE) and fluorography (Figure 6).

Centrifugation to equilibrium on potassium tartrate (50%)-glycerol (30%) gradients has been shown to be an important purification procedure. A profile of radiolabeled KAR #1 virus, PEG precipitated and centrifuged on a potassium tartrate-glycerol gradient for 14 hours at 40,000 rpm in a SW rotor is shown in Figure 7. The infectious virus peak is indicated by the arrow. Another peak of radioactivity was observed lower in the gradient (greater density). The PAGE profile of this lower band shows principally nucleocapsid protein as opposed to the virion band which contained the nucleocapsid polypeptide and at least 2 additional proteins (Figure 6). It was important to centrifuge potassium tartrate gradients to equilibrium because shorter time periods only separated the proteins on the basis of size (rate-zonal) Figure 8 depicts a gradient identical in composition to that shown in the previous figure but centrifuged for only 4 hours. The virion band is again indicated by the arrow but the more dense band of radioactivity lower in the gradient was considerably reduced.

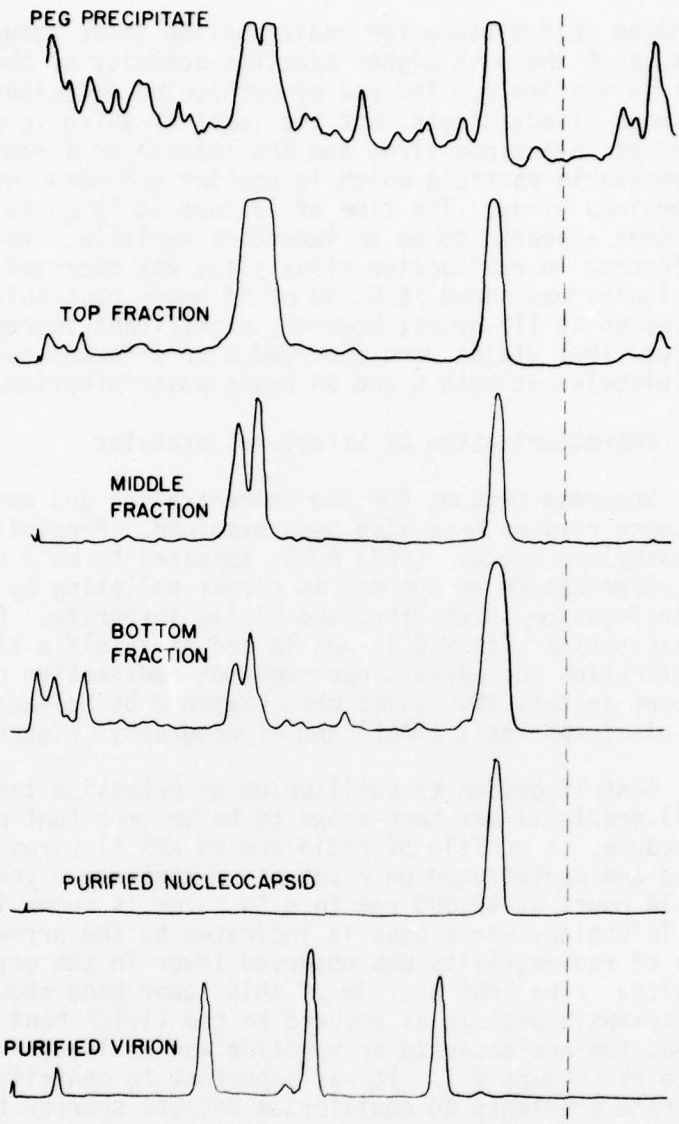


FIGURE 6. Densitometer tracings of 12% acrylamide-DATD discontinuous polyacrylamide gels developed by fluorography.

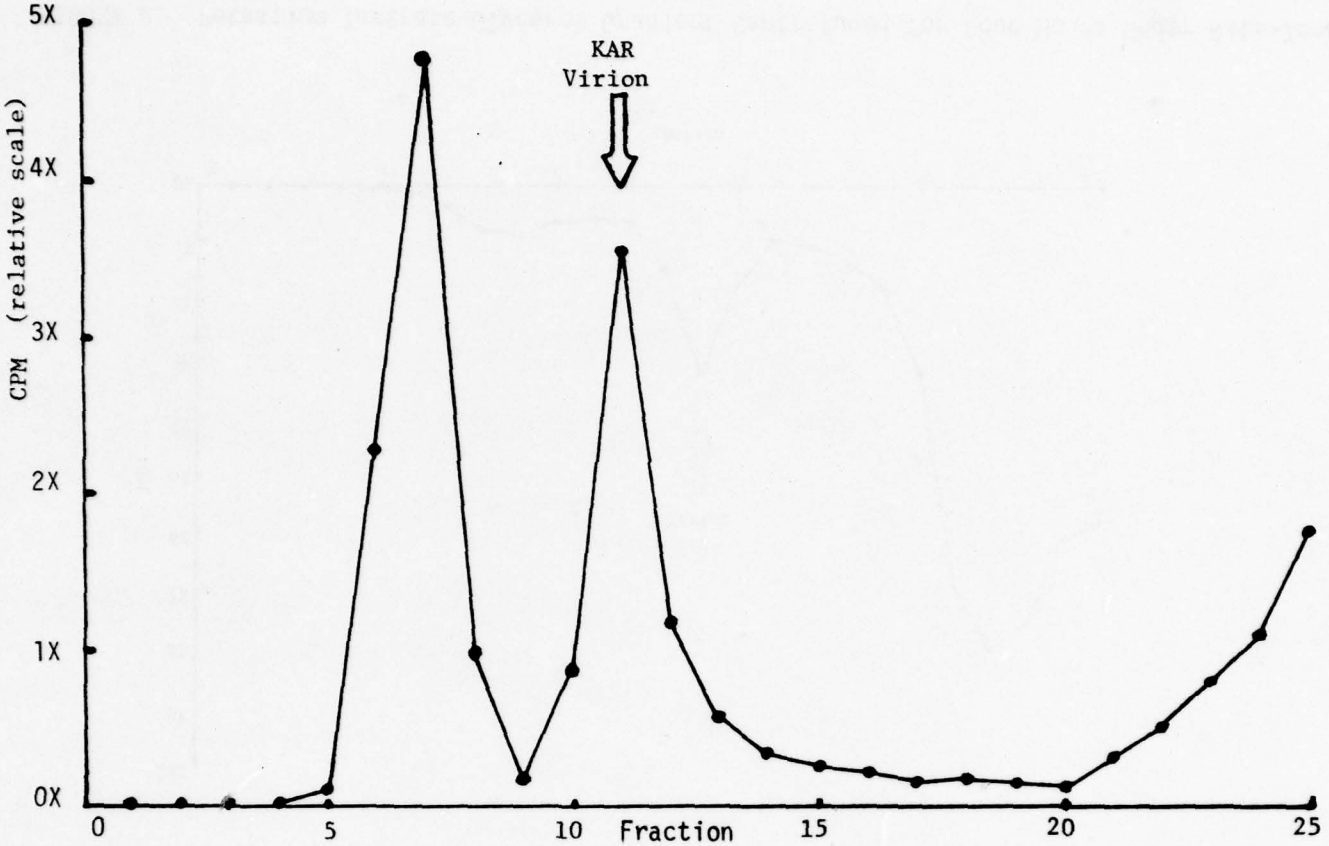


FIGURE 7. Potassium Tartrate-Glycerol Gradient Centrifuged to Equilibrium.

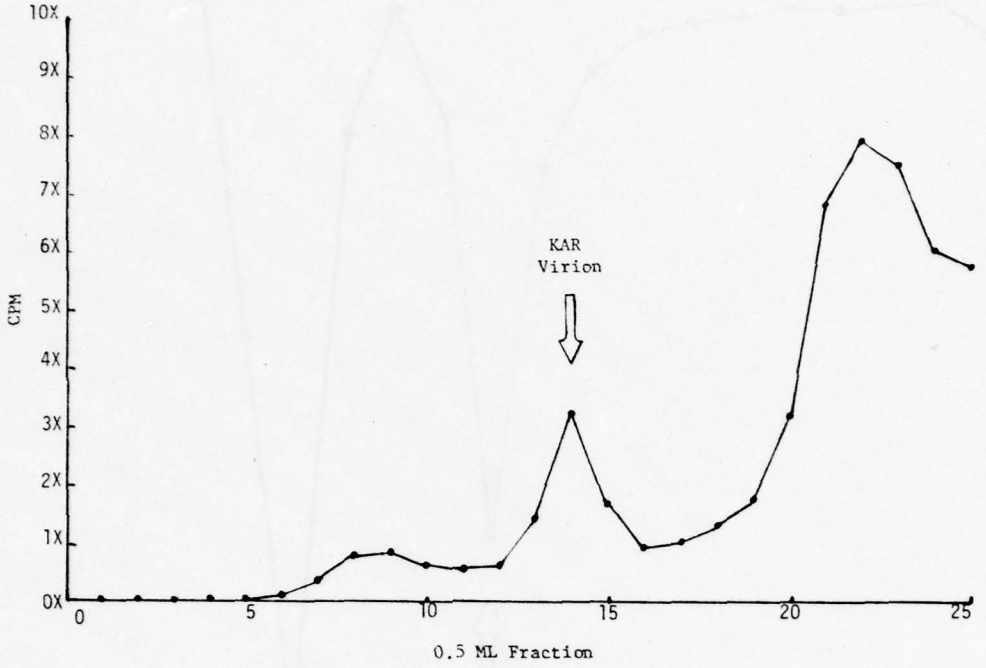


FIGURE 8. Potassium Tartrate-Glycerol Gradient Centrifuged for Four Hours Under Rate-Zonal Conditions.

Rate zonal centrifugation of the KAR #1 virion, nucleocapsid rich bands and a Sindbis virion marker is shown in Figure 9. These 10-40% sucrose gradients were centrifuged at 40,000 rpm for 60 minutes in the SW 41 rotor. These gradients clearly resolved the larger Karimabad virion from the smaller nucleocapsid protein rich fraction. The smaller size and greater density of this fraction is compatible with "core" particles that are either released in abundance into the supernatant fluid during infection or are the result of disruption of intact virions either during continued incubation with Vero cells or during the PEG concentration procedure.

Analysis of the radiolabeled virion polypeptides was accomplished using discontinuous PAGE on 12% acrylamide-diallyltartardiamide slabs. Samples were treated with 2% sodium lauryl sulfate (SLS) and 2% 2-mercaptoethanol (2-ME) at 100°C prior to electrophoresis. Radioactive polypeptides were detected by impregnating the dimethylsulfoxide dehydrated gel with fluor (PPO) and exposing the dried gel to x-ray film at -70°C. Using these procedures, the virion polypeptide profile, the nature of the various radioactive gradient fractions and the purity of preparations from various steps in virion purification could be monitored (Figure 6).

The densitometer tracings of fluorography films have been used for ease of illustration. The top tracing shows the complex mixture of radiolabebled proteins found in the PEG precipitate at an early stage in concentration. The second panel (Top Fraction) describes the virion fractions from a density centrifugation and the third panel (Middle Fraction) shows the additional purification achieved by a subsequent rate-zonal sucrose sedimentation. The dense nucleocapsid rich fractions from density centrifugation (Bottom Fraction) can be further purified by rate-zonal sucrose sedimentation as well to yield essentially clean nucleocapsid protein (Purified Nucleocapsid). The bottom panel (Purified Virion) shows the protein profile of Vesicular Stomatitis virus (VSV) included for purposes of marker proteins.

Although the reducing agent 2-mercaptoethanol (2-ME) was routinely used in the treatment of samples prior to PAGE, other laboratories working with these agents (D.H.L. Bishop, et al., Birmingham, AL) reported that the two larger envelope polypeptides could not be separated unless the reducing agent was either drastically reduced in concentration or eliminated entirely.

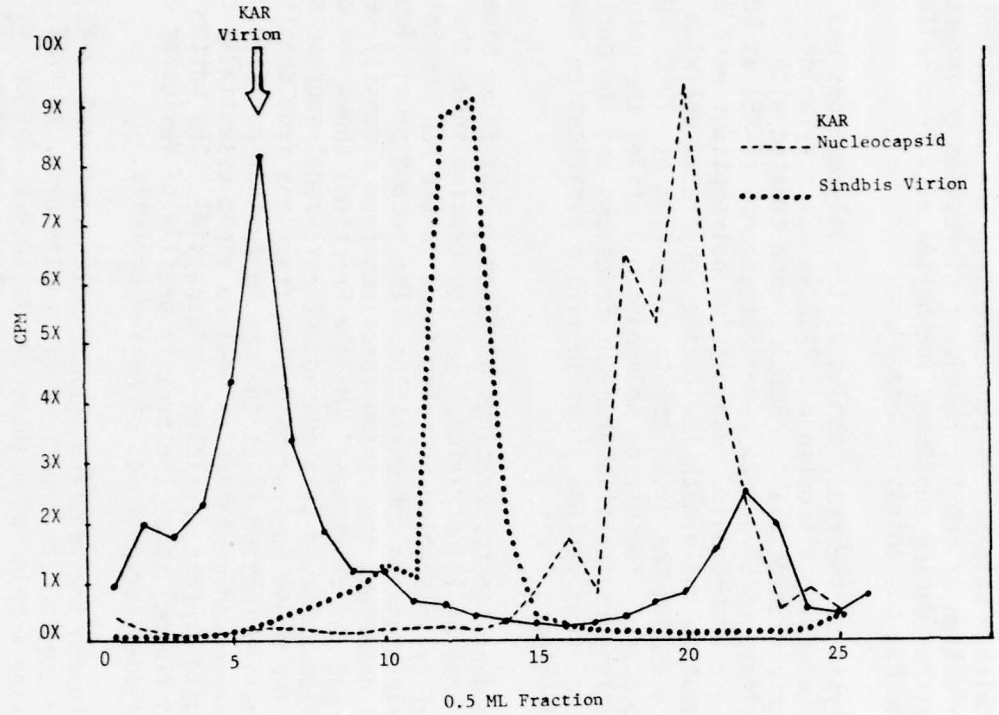


FIGURE 9. Rate-Zonal 10-40% Sucrose Gradient Centrifugation.

The results of an experiment testing various concentrations of 2-ME under similar PAGE conditions is shown in Figure 10. At high concentrations (2% 2-ME) the two polypeptides were resolved; however, the densitometer tracings did not show two distinct peaks. At lower concentrations these proteins were clearly not resolved yet the elimination of 2-ME entirely did show a disproportionate separation. It was interesting to note that decreasing concentrations of 2-ME allowed greater resolution of a small polypeptide migrating slightly faster than the nucleocapsid protein. Additional experiments are required to clearly describe the polypeptide composition of these viruses.

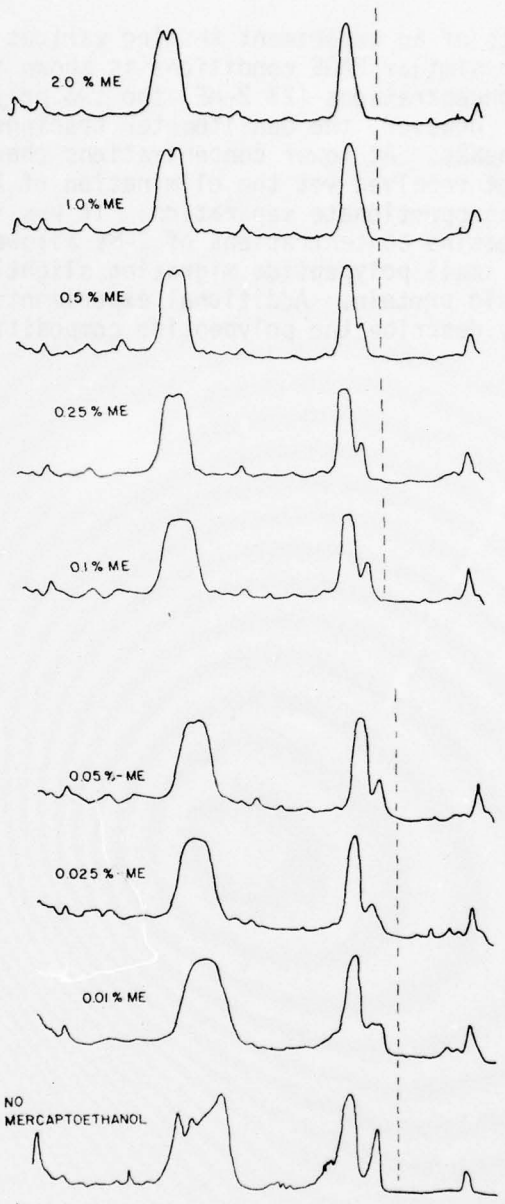


FIGURE 10. Desitometer tracings of 12% acrylamide-DATD discontinuous slab gels demonstrating the effect or prior treatment of ^{35}S-Karimabad virus with varying concentrations of 2-ME.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 097, Antigenic Characterization of Sandfly Fever Viruses

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0C6460	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NI	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	098
b. CONTRIBUTING				
c. XXXXXXXX				

11. TITLE (Procede with Security Classification Code)*

(U) Immunology of Plasmodium falciparum in vitro

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1	109
FISCAL YEAR CURRENT 79	1	155

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Tropical Medicine (U) Antigens (U) Antibodies (U) Immunity (U) Malaria

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23 (U) The objective of this Work Unit is to exploit newly developed methods of culture of Plasmodium falciparum to elucidate the mechanisms of immunity to the organism and explore the feasibility of development of immunological preventive methods against this major military infectious disease problem.

24 (U) The approach is to study the interaction of Plasmodium falciparum with immune serum and cells in vitro and to develop methods of antigen production and immunization

25 (U) 77 10-78 09 Studies have been conducted investigating the effect of serum from immunized owl monkeys on growth and development of Plasmodium falciparum in vitro. Owl monkeys were solidly immune after blood induced infections which were cured with chloroquine. Serially collected sera from these animals were evaluated for antibodies by immunofluorescence using a technique developed in this laboratory which employs the culture forms of P. falciparum as antigen. These studies demonstrate that maximum fluorescent antibody titers are obtained shortly after a boosting immunization with viable parasites and that such sera inhibit the development of the parasite in the in vitro assay. Development of the methodology for culture of malaria parasites has continued. Continuous flow devices, although theoretically highly desirable, involve many technical problems. Antigen for development of serologic tests has been collected by growth of parasites in large cell culture flasks with frequent medium changes. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 1977 - 30 Sep 1978.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 098 Immunology of P. falciparum in vitro

Investigators:
Jeffrey Chulay, MAJ, MC; Carter Diggs, COL, MC; Rufus Gore; Cynthia Hall, SP5; David Haynes, MAJ, MC; Lois Simonton; Andre Toussaint; Karen Wabnitz; Joseph Williams.

Objective: The objectives of this project are to improve the in vitro culture of the human malaria parasite Plasmodium falciparum and to use the cultured parasite in immunologic and biochemical studies elucidating the host-parasite relationship. The goal is to determine the feasibility of a vaccine against human malaria.

Description: The continuous culture of a malaria parasite has been possible only in the last two years. Using methods described in our laboratory (1), and at the Rockefeller University (2), we have been growing the Camp strain of P. falciparum for periods of months without interruption. We concentrated on the Camp strain because it had been well adapted to and extensively studied in the Aotus monkey here at WRAIR. Considerable data has been accumulated about the Aotus' immune response to this strain, and immune serum had been collected which can passively protect in vivo against this parasite. However, our attempts to improve culture conditions and scale up growth have have thus far failed. The growth rate of this strain under our standard conditions was so variable that attempts to evaluate new conditions often failed for lack of a satisfactory control; and only sporadically were we able to achieve parasitemias above 3%. Two lines of evidence suggest that this parasite is unusually sensitive to inhibition of growth by several agents (see below). We have recently obtained two other P. falciparum strains from other laboratories and have confirmed that these grow much better than the Camp strain using our standard conditions. We therefore plan to concentrate most of our efforts at increasing parasite yields with these two strains. We will continue to use the Camp strain for some studies of immunity in Aotus, and for antigenic comparison with the other strains. Working the past year with the Camp strain, our lab has nevertheless made several useful observations, including the use of cultured P. falciparum as the antigen in a malaria indirect fluorescent antibody test (3), and the development of a microtiter well assay for immune serum inhibition of parasite growth and reinvasion (4). In the past year, other laboratories have described methods which seem promising for (a) inducing synchrony

so that the mature parasite stages which are thought to be antigenically most important can be obtained in greater purity; and (b) concentrating the parasitized red cells (a first step in antigen purification). However it is becoming more apparent that the mature parasite (eg. merozoite) is a self-destructive organism (personal communications from several labs), making many techniques of antigen analysis impossible at present. This self-destruction probably is one of the reasons that such strong adjuvants have been necessary to elicit a protective immune response to vaccination with merozoites. Therefore one of our high priorities is to find methods for stabilizing the mature forms of the parasite.

Progress: The erratic growth of the Camp strain of P. falciparum in culture has severely hampered the development of methods to analyse malaria antigens, and the immune responses to these antigens. There were a few periods of consistent growth (Fig. 1), but by the middle of FY 78 growth had become increasingly erratic. Often the parasites grew well during the weekend, but when we attempted to use them during weekdays, their growth rate diminished markedly (Table 1). About this time, we noted that the fumes from the connecting histology lab were becoming more frequently noticeable and often during the weekdays were so strong in our lab that several investigators would become ill. In June 1978 at a malaria immunology and culture conference, it was learned that two other labs had had similar problems with the exoerythrocytic culture stages of avian malaria, which were solved only when the sources of fumes were removed. A short trial of culturing our parasite in another lab indicated that improved growth could be obtained, and after exploring the options it was decided to move the malaria culture and immunology work to another laboratory. This occurred during the summer of 1978. The Camp strain is now growing more consistently, but still does not attain the growth rates and parasitemias reported by other groups using other strains.

In order to determine whether the limiting factor now was our technique or the strain, two vigorous strains from other laboratories were obtained and cultured simultaneously with our standard technique, and with the techniques of the labs of their origins (the Z strain was obtained from the NIH, and the FCR-3 strain from the Rockefeller University). These experiments indicated that our basic technique was good, but that serum supported better growth of these strains than did our previously standard fresh frozen plasma (however with the Camp strain plasma seemed to be equally as good as serum). Presently

we are achieving parasitemias of 5 to 10%, with growth rates of 7 to 10 fold every two days, by culturing the FCR-3 strain parasite in Corning tissue culture flasks containing 6% human erythrocytes v/v in 10% human serum-25 mM HEPES buffered-RPMI 1640 gassed with 5% carbon dioxide, 5% oxygen, 90% nitrogen, and incubated at 37 C. Under the same conditions, the Camp strain achieves parasitemias of 2 to 4%, with growth rates of 3 to 7 fold every two days. The higher parasitemias are achieved with twice daily media changes.

Previous work with cultures of the Camp and Z strains resulted in a paper describing their use as antigen in a malaria indirect fluorescent antibody (IFA) test (ref. 3 and Table 2). Recently, cultured parasites have been concentrated by a Ficoll sedimentation technique and successfully used as a crude antigen in a microtiter ELISA test for antimalaria antibody.

Further evidence for the unusual sensitivity of the Camp strain to inhibitory influences comes from a series of experiments looking at the effect on growth rate of storage of the erythrocytes (as CPD or ACD anticoagulated whole blood) for varying periods at 4 C. Previously, Jensen had shown that blood stored for 21 days was superior to fresh blood (5). We have similar findings, but in addition find that blood stored from 2 to 7 days is very poor at supporting the growth of the Camp strain (Fig. 2). We have not yet repeated this with other strains, but personal communications from other labs indicate that the growth of other strains of parasites is not as much inhibited by blood stored for 3 days.

Four experiments were done with the Camp strain to determine optimal pH and osmolarity (Figs. 3 and 4). Using a dilute suspension of red cells and parasites grown for 48 hours in microtiter wells, then pulsed for 18 hours with tritiated-hypoxanthine, and harvested with a MASH, it appeared that the parasite could tolerate a moderate range of pHs and osmolarities, and that our standard medium with a pH of about 7.4 and osmolarity of about 305 mOsm was in the middle of both ranges.

Further investigation of the suitability of tracer labelled polynucleotide or protein synthesis as an index of parasite number showed that tritiated hypoxanthine and leucine uptake were each susceptible to a number of influences, including stage of the parasite in its growth cycle, number of uninfected red cells present, amount of competing cold isotope present (may be introduced in varying amounts by different sera), and the health of the parasite

(eg. as influenced by time between media changes). Nevertheless, under appropriately controlled conditions, tracer incorporation correlates well with numbers of parasites (Figs. 5 and 6).

In the microtiter plate drug screening assay (6) inhibition of parasite growth as determined by tritiated-hypoxanthine incorporation closely parallels inhibition of parasite growth as determined by morphology (Fig. 7).

Aotus monkeys immunized with the Camp strain by infection and drug cure are resistant to further infection with that parasite. Figure 8 shows the course of one such animal initially infected with one million parasitized erythrocytes, and subsequently immune to challenge with as many as 400 million parasitized erythrocytes (cryopreserved from its own previous infection and shown to be infectious in another monkey). The IFA titer of the monkey's serum rose following the initial infection, and was boosted by the subsequent challenge. Sera taken from these monkeys when the IFA titer was high was heat inactivated and incorporated into microtiter well cultures of the parasite, and after 2 to 4 days, the amount of growth was assessed morphologically. Pre-immune sera often enhanced growth, whereas immune sera consistently decreased growth (4). The effect was more marked and could be titered out further in the 4 day cultures (Fig. 9).

Discussion: Although much has been left undone because of the problems with growth of the Camp strain, now that laboratory conditions are more satisfactory, and other strains have been obtained which are more vigorous in culture, more rapid progress may be hoped for in the analysis of parasite antigens and their relationships to protective immune responses. Amounts of parasite produced can be scaled-up by using larger flasks and manually changing the medium more often, however attempts to develop an automated flow system are not being abandoned. Particular attention will be paid to the assessment of methods for synchronizing and purifying the mature stages of the parasite. Methods of stabilizing the merozoite are being sought. Techniques for immunochemical evaluation of parasite antigens are being pursued. Arrangements have been made to visit a leading laboratory in the field of serum-free culture, so that more rapid progress may be made toward independence from human serum, which in addition to its other problems, is in short supply. Other assays for in vitro correlates to protective immunity are being investigated.

Legends for Figures

Figure 1. Growth of P. falciparum (Camp strain) in culture medium 199 with 15 mM TES buffer and 10% fresh frozen human plasma (heat inactivated).

Figure 2. Effect of storing red cells in ACD or CPD at 4C for varying periods of time. Growth of the Camp strain for four days in medium RPMI 1640 with 25 mM HEPES buffer and 10% fresh frozen plasma, 6% Hct, 5 ml per 25 sq cm flask, gassed with 5% carbon dioxide, 5% oxygen, 90% nitrogen. Medium (4 ml per flask) changed daily. Data from six experiments representing blood from 3 donors.

Figure 3. Effect of initial pH on parasite growth (Camp strain) as evaluated by tritiated-hypoxanthine incorporation. 18 hour pulse of 0.5 µCi after 48 hour pre-incubation of 0.1% parasitemia, 1% Hct (x), or 0.25% Hct (o), 250 µl per microtiter plate well, in triplicate. Grown at 37C in a box gassed with 5% carbon dioxide, 5% oxygen, and 90% nitrogen. Harvested with distilled water onto glass fiber filter paper with a multiple automated sample harvester (MASH) apparatus, and counter in scintillation fluid. Data from two experiments with osmolarity kept constant at 300 mOsm. The pH was adjusted with varying amounts of NaCl and NaHCO3 solutions, and measured after equilibration with 5% carbon dioxide on a Corning blood gas machine at 37 C.

Figure 4. Effect of osmolarity on parasite growth with pH held constant at 7.4. The osmolarities were adjusted with NaCl solutions and measured by freezing point depression. Conditions otherwise were as stated for figure 3. The standard medium has a pH of 7.37 and an osmolarity of 300 mOsm.

Figure 5. Correlation of tritiated-hypoxanthine incorporation with morphologic determination of parasite number at time zero. All cultures in triplicate microtiter plate wells, 1% Hct, 200 µl, were pulsed with 1.5 µCi for 18 hours, after an initial pre-incubation of 0, 24, or 48 hours. Harvested with distilled water and MASH apparatus.

Figure 6. Correlation of tritiated-leucine incorporation with morphologically determined parasite number at time zero. Leucine-free RPMI 1640, 1.5% Hct, 0.5 µCi tritiated-leucine pulse, normal saline harvest. Conditions otherwise the same as stated for figure 5.

Figure 7. Comparison of drug inhibition of parasite growth assayed morphologically and isotopically. Serial two-fold dilutions of drug (in this case chloroquine from 139 to 2.2 ng/ml) were made in 25 µl media per well. Quadruplicate cultures of 0.5% parasitemia (Camp strain), 1.5% Hct, 200 µl per well were added and incubated as previously described. At 24 hours, duplicate wells were harvested for morphological determination of parasitemia, and duplicate wells were pulsed with 0.5 µCi tritiated-hypoxanthine. After another 18 hours, the pulsed cultures were harvested with distilled water and a MASH apparatus, and the incorporation determined by scintillation counting. The effective dose of drug to cause a 50% inhibiton (ED-50) was calculated by a nonlinear computer analysis.

Figure 8. Infection (Camp strain) followed by drug cure protects an Aotus monkey against further parasitemia when challenged with parasitized autologous erythrocytes (PE). The same PE caused a fulminant infection in a non-immune monkey. Serum anti-malaria antibody was detected by an indirect fluorescent antibody test using fluorescein conjugated rabbit anti-Aotus IgG, and cultured parasites as antigen. Similar results were obtained in two other monkeys.

Figure 9. Immune serum inhibits parasite growth in a microtiter plate assay. Aotus sera were taken before (non-immune), or 10 weeks after (immune) chloroquine drug cure of a Camp strain infection. Sera were heat-inactivated (56 C for 30 minutes) and then serially diluted into triplicate cultures of the Camp strain (0.2% parasitemia in 0.8% Hct type O human erythrocytes, media as in figure 2, 250 µl per well). Media wash changed (200 µl per well) daily for three days and cultures harvested for morphological evaluation on day 4. Similar but slightly less dramatic results were obtained with 2 day assays. A satisfactory isotopic method has not yet been devised because different sera have different effects on tritiated-hypoxanthine uptake sometimes discordant with their effects on morphological growth.

Table 1

Growth of P. falciparum after 4 months in culture.

Days of the week	Highest Parasitemia	Multiplication rate per 48 hour cycle
Wednesday- Friday	0.2%	2.3
Friday-Monday	0.8%	6.2
Monday-Friday	0.4%	2.8
Friday-Tuesday	1.6%	11.6
Tuesday-Friday	0.1%	1.2
Friday-Monday	1.0%	5.6

Table 2

Indirect fluorescence antibody test using two strains of cultured P. falciparum as antigen

serum	IFA titer using Camp strain	Z strain
reference pos.	320	320
African 1	320	320
African 2	5,120	1,280
control 1	<10	<10
control 2	10	10

Figure 1

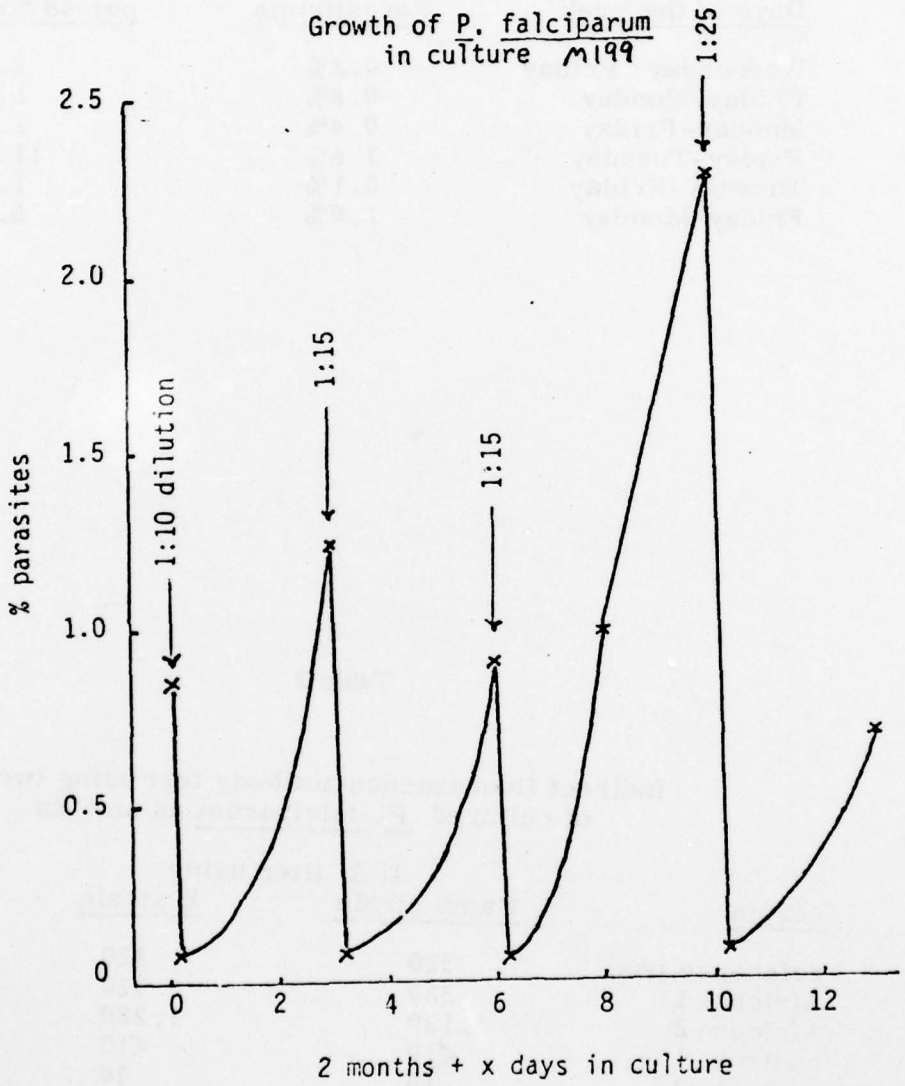
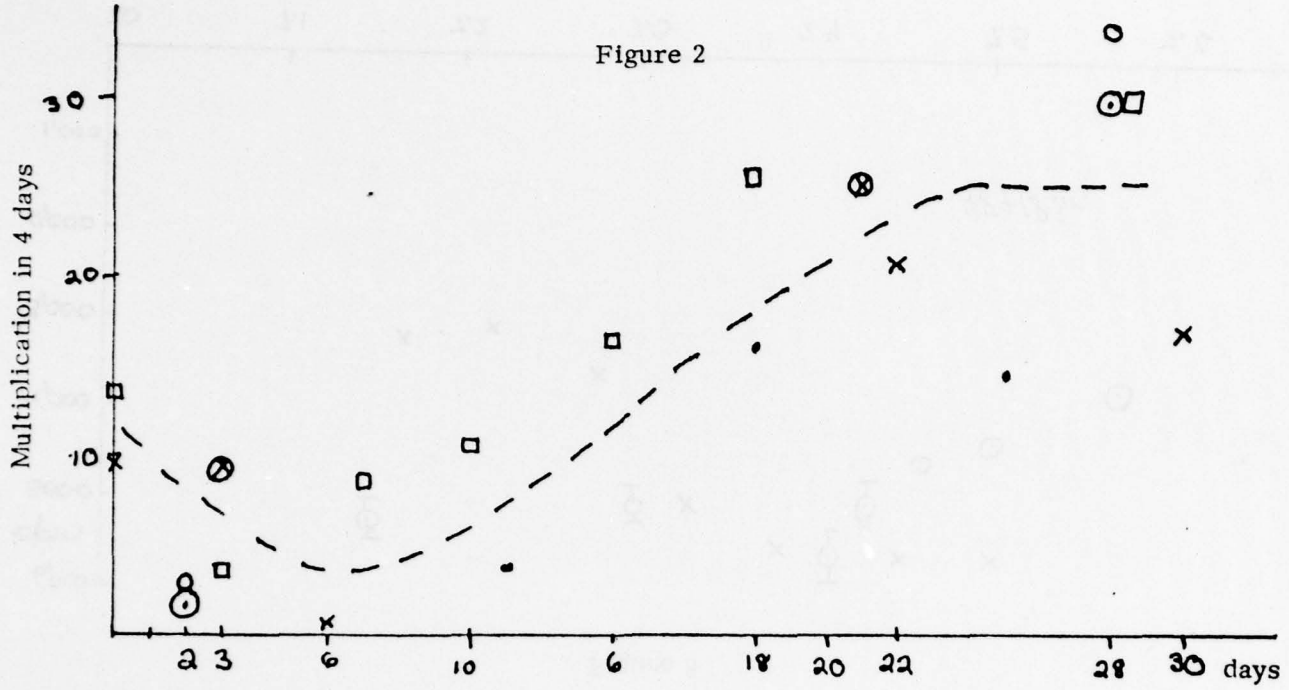
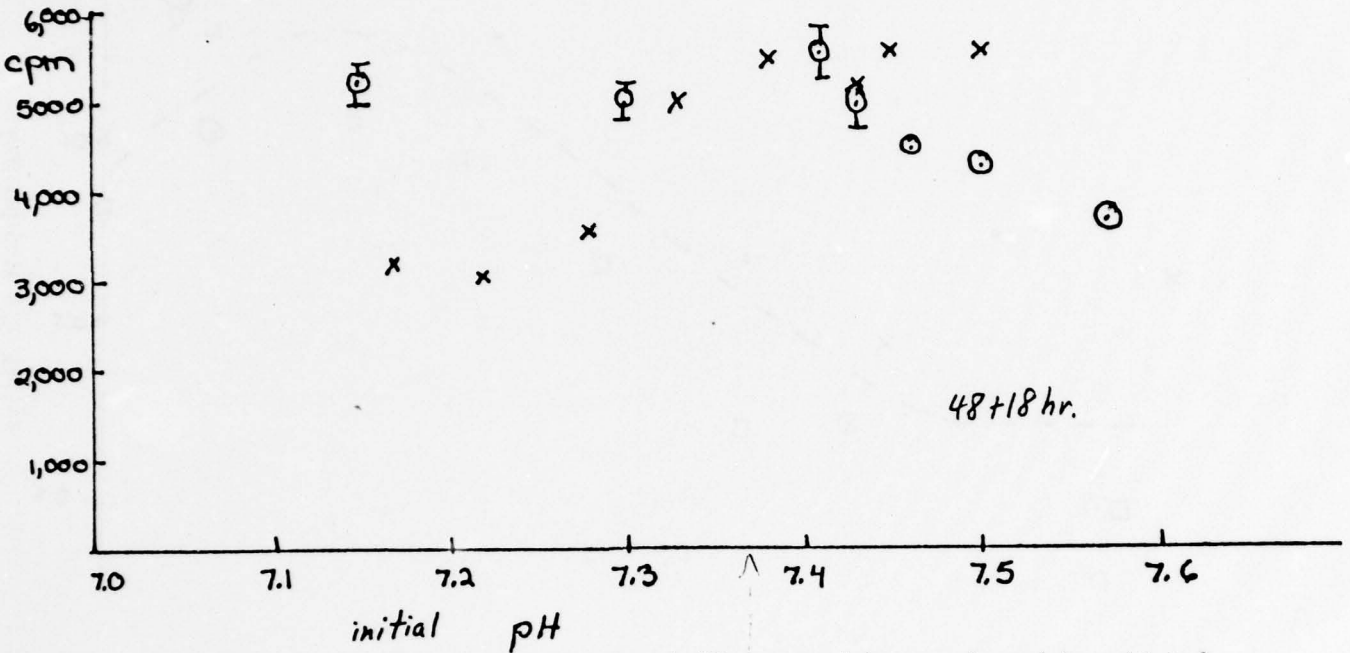

Growth of P. falciparum
in culture M199
% parasites
2.5
2.0
1.5
1.0
0.5
0
1:10 dilution
1:15
1:15
1:25
0
2
4
6
8
10
12
2 months + x days in culture

Figure 2



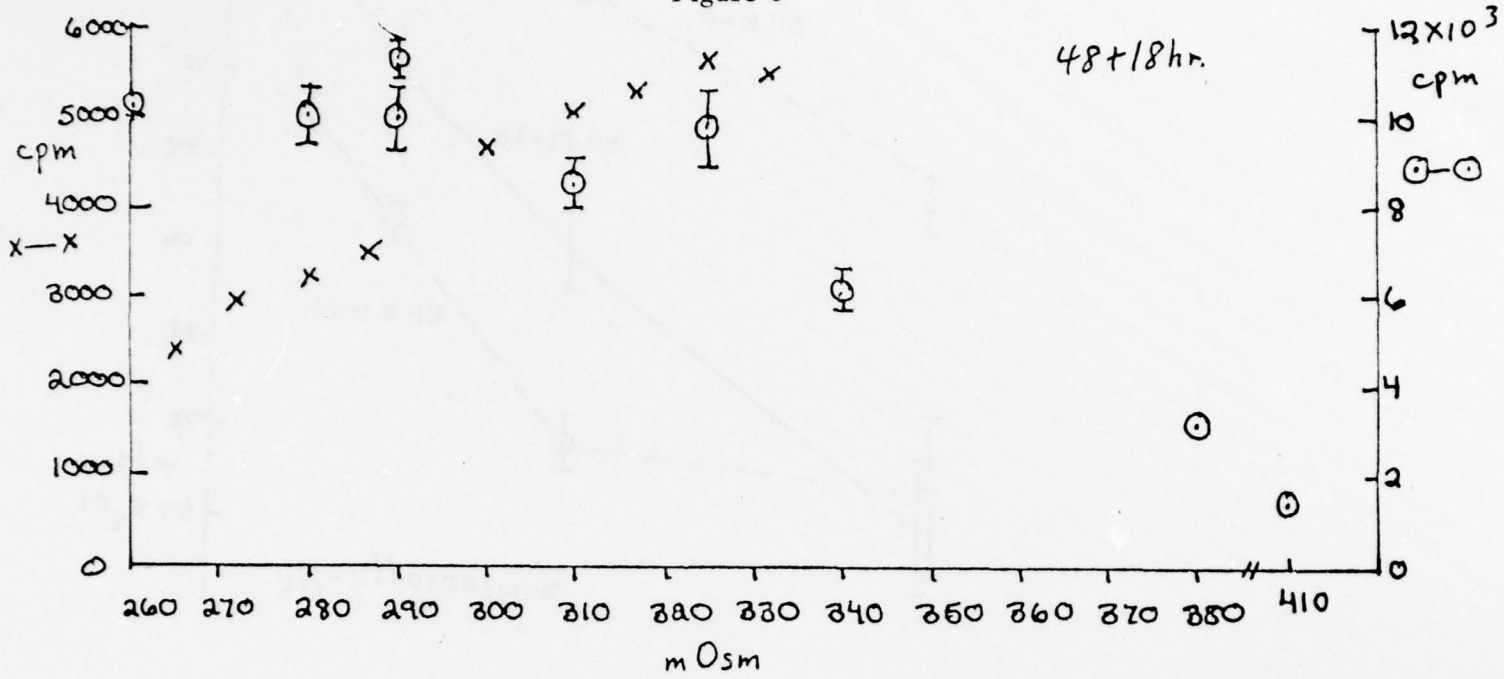
RBC stored x days at 4° C in ACD or CPD before use in culture.

Figure 3



Effect of initial pH on parasite growth (Camp strain) as evaluated by tritiated-hypoxanthine uptake (18 hr. pulse after 48 hr. pre-incubation).
Two experiments, 300 mOsm.

Figure 4



Effect of osmolarity on parasite growth (Camp strain) as evaluated by tritiated-hypoxanthine uptake (18 hr. pulse after 48 hr. pre-incubation).
Two experiments, pH 7.4.

AD-A074 241 WALTER REED ARMY INST OF RESEARCH WASHINGTON DC F/G 6/5
RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMIS--ETC(U)
1978

UNCLASSIFIED NL

2 OF 8
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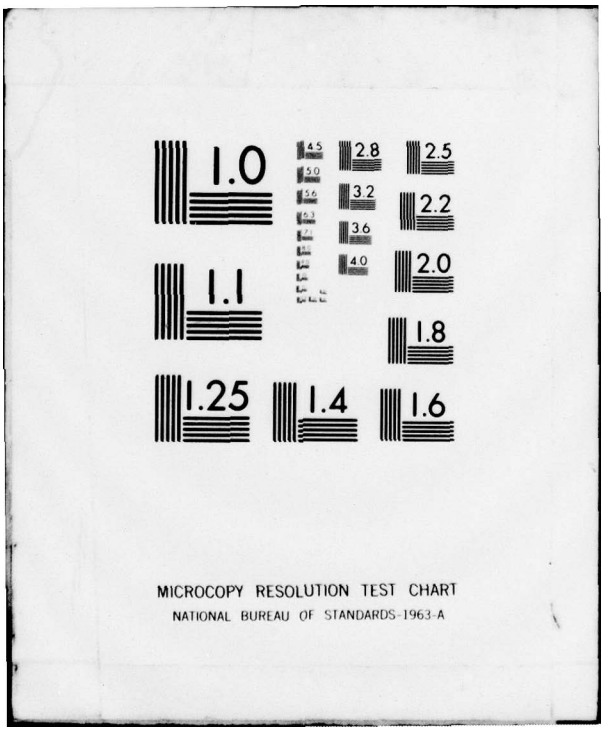

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

Figure 5

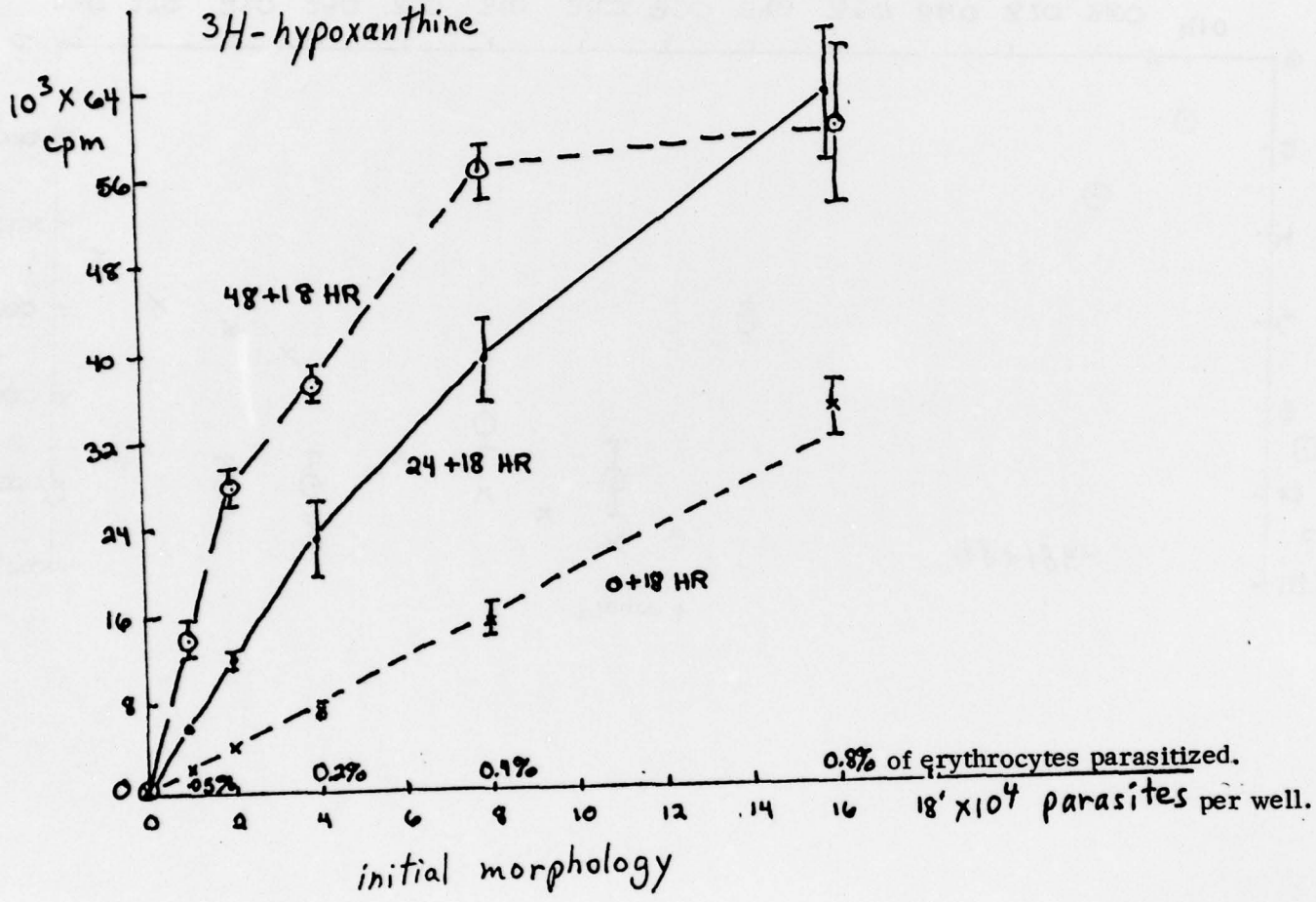

3H-hypoxanthine
10^3 X 64 cpm
56
48
40
32
24
16
8
0
48+18 HR
24+18 HR
0+18 HR
0.2%
0.9%
0.8% of erythrocytes parasitized.
0
2
4
6
8
10
12
14
16
18' X 10^4 parasites per well.
initial morphology

Figure 6

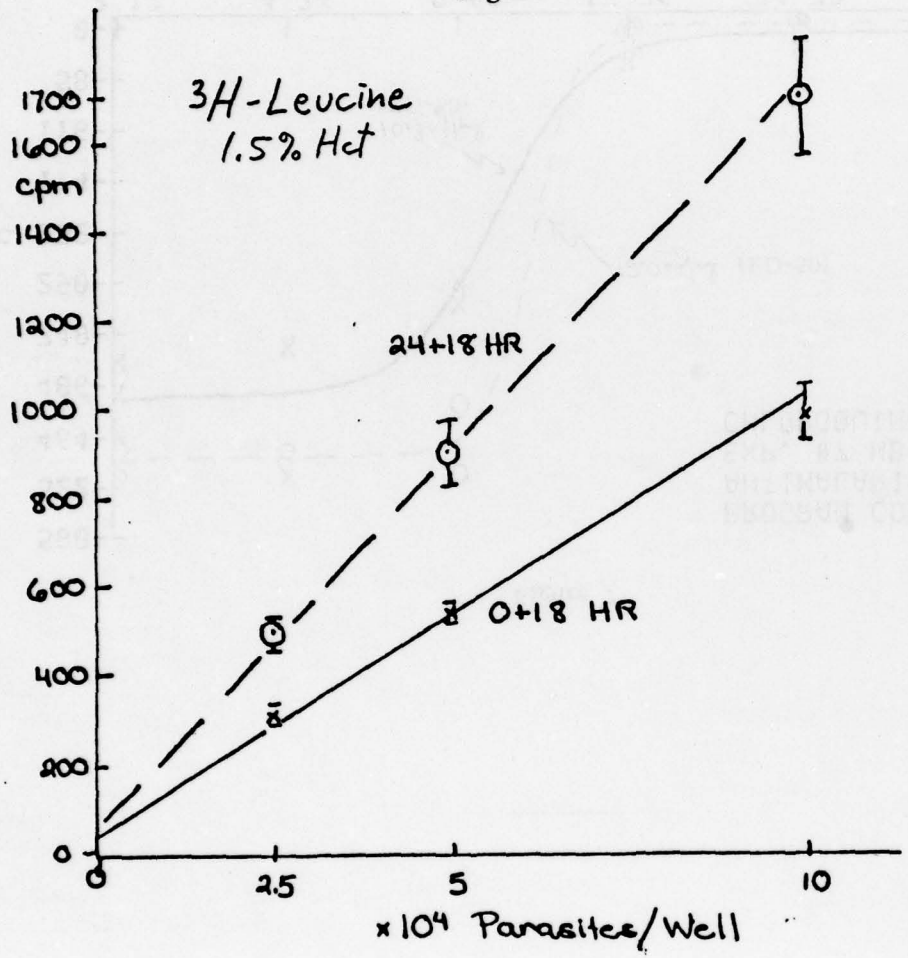

3H-Leucine
1.5% Hct
1700
1600
cpm
1400
1200
1000
800
600
400
200
0
24+18 HR
0+18 HR
0
2.5
5
10
x 10⁴ Parasites/Well

Figure 7

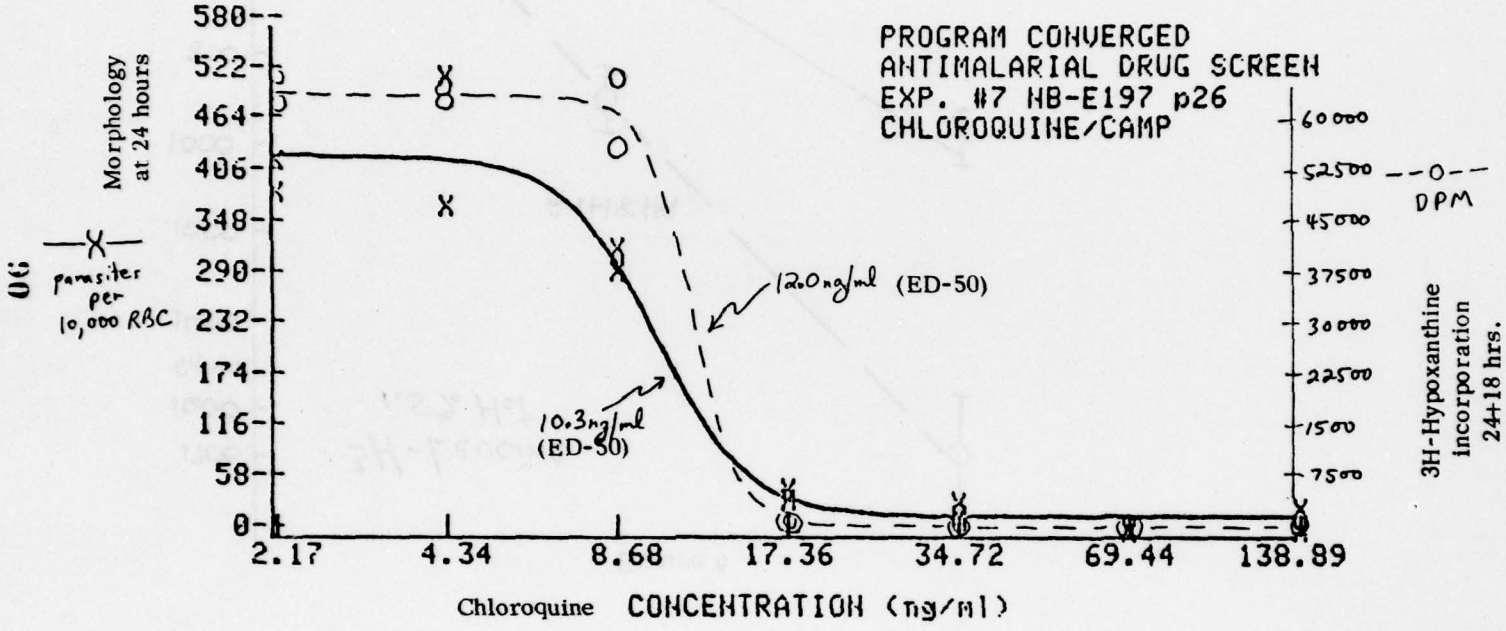

PROGRAM CONVERGED
ANTIMALARIAL DRUG SCREEN
EXP. #7 NB-E197 p26
CHLOROQUINE/CAMP
Morphology at 24 hours
parasites per 10,000 RBC
3H-Hypoxanthine incorporation 24+18 hrs.
DPM
120 ng/ml (ED-50)
10.3 ng/ml (ED-50)
580
522
464
406
348
290
232
174
116
58
0
60000
52500
45000
37500
30000
22500
15000
7500
2.17
4.34
8.68
17.36
34.72
69.44
138.89
Chloroquine CONCENTRATION (ng/ml)

Figure 8

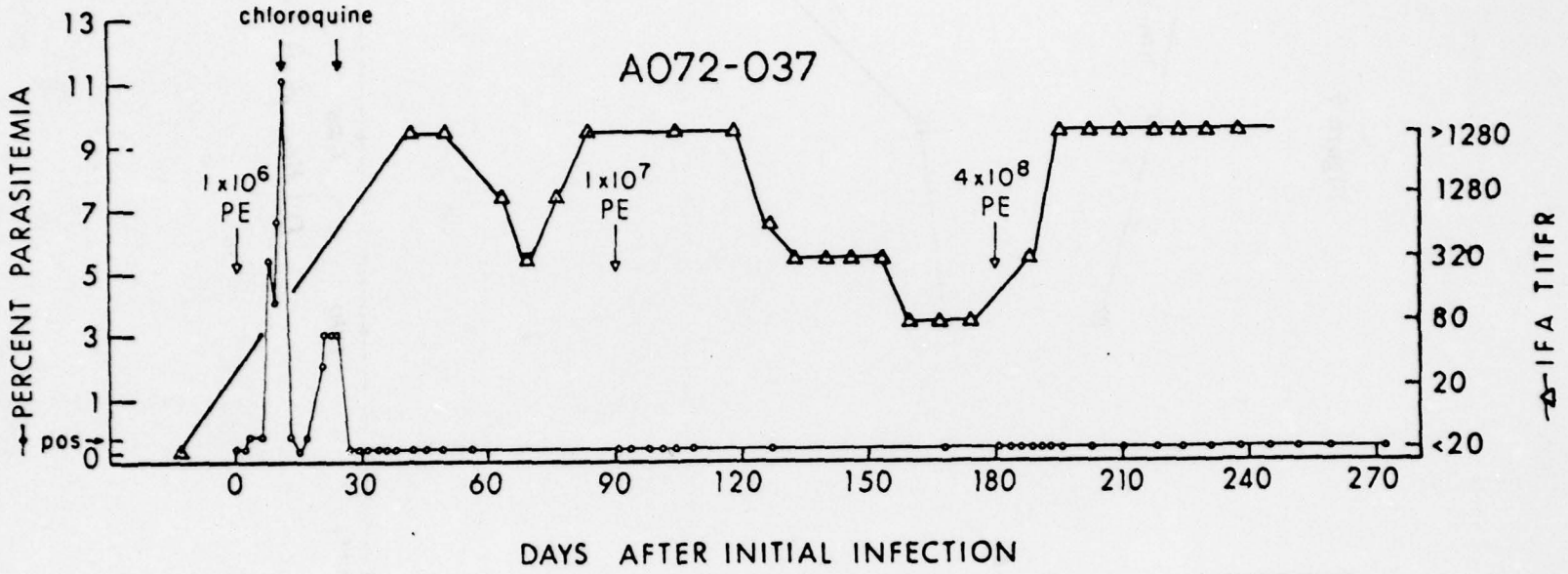
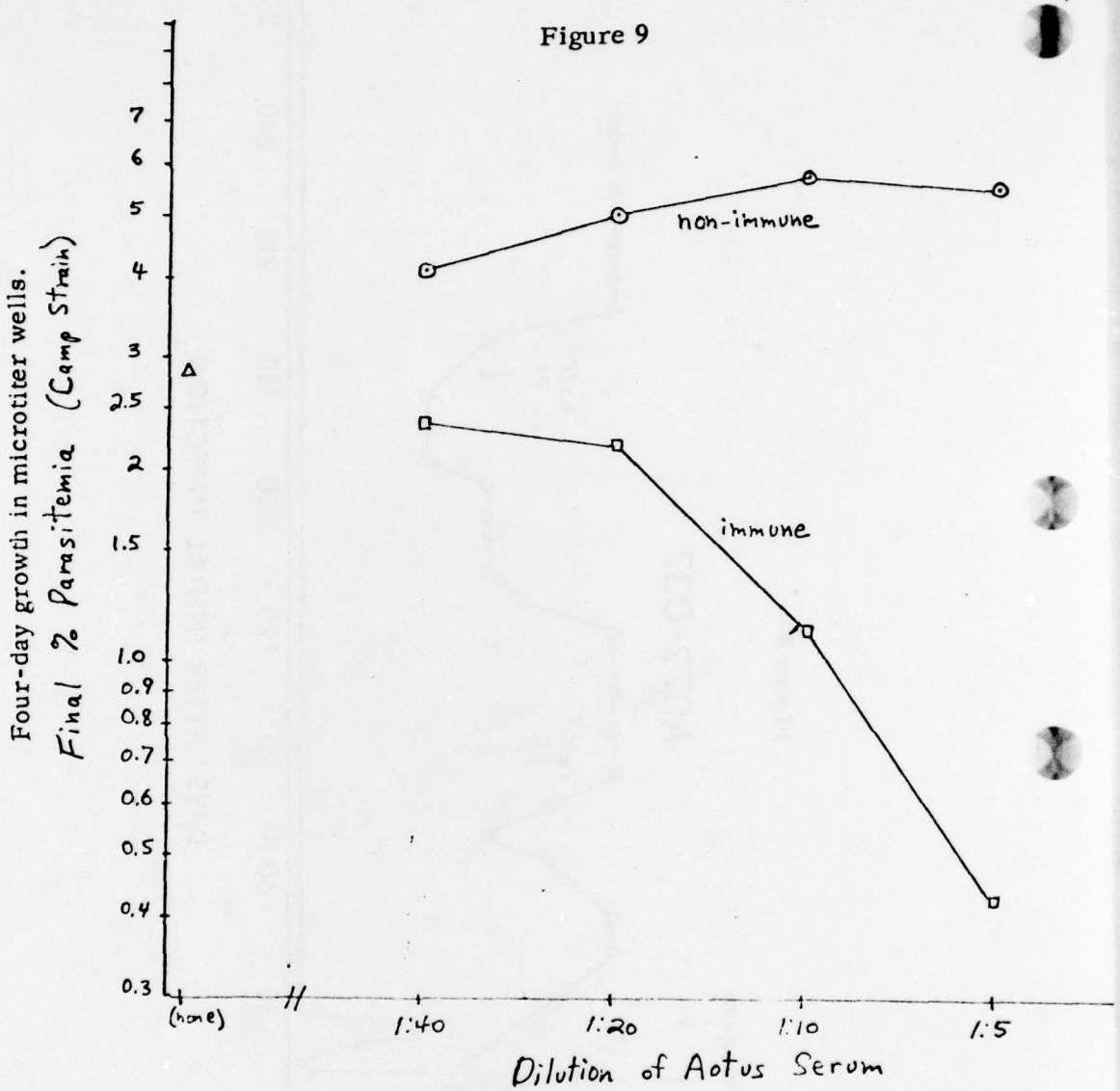

chloroquine
A072-037
1x10^6 PE
1x10^7 PE
4x10^8 PE
PERCENT PARASITEMIA
pos
13
11
9
7
5
3
1
0
>1280
1280
320
80
20
<20
IFA TITER
0
30
60
90
120
150
180
210
240
270
DAYS AFTER INITIAL INFECTION

Figure 9


Four-day growth in microtiter wells.
Final % Parasitemia (Camp Strain)
7
6
5
4
3
2.5
2
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
non-immune
immune
(none)
1:40
1:20
1:10
1:5
Dilution of Aotus Serum

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 098 Immunology of P. falciparum in vitro

Literature Cited:

References:

1. Haynes, J.D., Diggs, C.L., Hines, F.A., and Desjardins, R.E., 1976. Culture of human malaria parasites Plasmodium falciparum. Nature, 363: 767-769.

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Publications:

References 3, 4, and 6.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6461	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M762770A803		092
b. CONTRIBUTING	61101A	3A161101A91C		099
c. XXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Chemotherapy and Chemoprophylaxis of Leishmaniasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	Cont.	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 78	1.4	99
FISCAL YEAR — CURRENT 79	2.0	150

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Rapmund, G., COL
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of Experimental Therapeutics
ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Davidson, D.E., Jr., LTC
TELEPHONE: 202-576-2292
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Hendricks, L.D., MAJ
NAME: Childs, G.E., CPT

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Leishmaniasis; (U) Drug Development; (U) Biology; (U) Chemistry; (U) Toxicology

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against the cutaneous and visceral forms of leishmaniasis, a parasitic infection of the reticuloendothelial system, which poses a serious hazard of disability, disfigurement and death to military personnel operating in most of the tropical and subtropical regions of the world, including Latin America, Asia, Africa and the Near East. The only drugs currently available are antimonials, which are hazardous to use and frequently ineffective.

24. (U) Selected chemical compounds from the malaria drug inventory will be tested for antileishmanial activity (1) in vitro, (2) in laboratory rodents, (3) in subhuman primates or canines. A series of leishmania models which have been developed or modified by this laboratory will be used to test candidate antileishmanial drugs: (1) Axenic and cell cultures of Leishmania braziliensis amastigotes, (2) L. donovani in hamsters, (3) L. tropica in rodents, (4) L. braziliensis in Mystromys albicaudatus rats, (5) L. donovani in Macaca fascicularis, (6) L. donovani in canines.

25. (U) 77 10 - 78 09. Miniaturization and establishment of dose response curves for known active antileishmanial drugs have been achieved for the in vitro drug screening system. Work continues on further miniaturization and automation of this system. The histological studies of the L. braziliensis/Mystromys model have been completed. The Mystromys has demonstrated a susceptibility to L. mexicana and data indicates that this system may be useful for prophylactic screening. Liposome encapsulated drugs have demonstrated markedly increased efficacy in rodents and work is progressing to pursue these leads in advanced animal models. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 099 Chemotherapy and Chemoprophylaxis of Leishmaniasis

Investigators.
Principal: MAJ Larry D. Hendricks
CPT George C. Childs
Associate: CPT LuAnn McKinney
LT CMDR Erich Stafford

I. IN VITRO DRUG SCREENING USING CULTURES OF AXENIC AMASTIGOTES.

An in vitro system for culturing leishmanial amastigotes free of host cells has been adapted for screening candidate anti-leishmanial drugs. Promastigotes of Leishmania braziliensis are grown in Schneider's Drosophila medium at 27°C, and transformed to amastigotes by a change to Medium 199 and temperature elevation to 34°C. These axenic amastigotes are then exposed to graded concentrations of control and candidate drugs. The treated amastigotes are washed and again transferred to Schneider's medium at 27°C. Failure of these amastigotes to convert and grow out as promastigotes is indicative of drug activity. Data on the effects of standard and experimental drugs such as the pentavalent antimonials, Glucantime and Pentostam, at various exposure times are being accumulated to characterize and standardize the test. Current efforts include evaluation of automated cell counting techniques to support this drug screening system.

Very little information is available to establish whether the regional differences in response of leishmaniasis to chemotherapy are due to natural geographic variation in the parasite or its host, or whether they are due to development of resistance from repeated ineffective drug treatment. Initial experiments indicate that the axenic amastigote culture system may also be useful for demonstrating and studying drug resistance in various strains or species of Leishmania. Priority efforts are underway to explore this particular approach.

Investigations of L-glycyl-L-histidyl-L-lysine as a chemically defined substitute for fetal calf serum (FCS) in Schneider's medium and Medium 199 used for the amastigote culture system have been completed. Low concentrations of this material (40 nanograms/ml) are sufficient to support rapid growth of promastigotes of several species of Leishmania; however, promastigotes cultured in Schneider's medium with the serum substitute failed to convert to viable amastigotes when placed in Medium 199 at 37°C with either L-glycyl-L-histidyl-L-lysine or 30% FCS. These

results suggest that the substitute may lack an essential component necessary to support the promastigote-amastigote conversion, and thus this substance will not permit use of a chemically defined media for the axenic amastigote system. However, with this chemically defined serum replacement, we now have a system for the cultivation of mass quantities of Leishmania promastigotes in a commercially available defined medium. Such organisms will be useful for antigenicity, immunological, metabolic or similar studies in which large numbers of organisms and a defined media are essential.

Collaborative efforts with Drs. Shiigi and Mishell, Department of Bacteriology and Immunology, University of California, Berkeley, to identify the Leishmania growth factor(s) provided to various lots of FCS by growth of a cryophilic bacterium are continuing.

II. ANIMAL MODELS OF LEISHMANIASIS.

A. Mystromys albicaudatus (African white-tailed rat)

Evaluation of the infection of L. braziliensis on M. albicaudatus as an animal model for cutaneous leishmaniasis has continued. An investigation of the gross and histological pathology of Leishmania braziliensis infections from the time of infection through 12 weeks has been completed. This study involved 54 animals with groups of infected animals and inoculum vehicle controls sacrificed at 24, 48, 72 hrs, and then sequentially on a weekly basis through 12 weeks.

Grossly, the lesion started as a raised nodule that continued to increase in size and then ulcerate. Scarring of the lesion occurred subsequent to bacterial contaminations. Microscopically, the lesion initially exhibited an acute exudative inflammation that progressed to a local chronic granulomatous dermatitis. The granulomatous stage was severe, with cellulitis, exudation, and massive infiltration of neutrophilic cells. The granulomatous lesion also contained numerous histiocytes containing myriads of developing Leishmania amastigotes. As the lesion progressed the occurrence of epitheloid cells with peripheral fibroplasia characterized the tissue response. In some cases satellite lesions were formed from small foci of infected histiocytes. The progression of these satellite lesions mimicked the development of the "parent" granulomatous lesion. In a few cases in which a regression of the lesion was noted, an infiltration of small lymphocytes was observed at the margins of the lesion. The progression of these lesions--in particular, the

sequence of cellular responses associated with their development—parallels closely the sequence as reported from human infections.

The long life span of Mystromys, up to five years, may enable it to be used in a model to study long-term chronic infections. Studies to investigate the pathogenesis of infection with a human mucocutaneous isolate over a three year period have been initiated. Such studies may provide important information on the metastatic process which leads to mucocutaneous manifestations in man.

Infections of Mystromys with L. braziliensis have been utilized for testing therapeutic candidate antileishmanial drugs, e.g., Glucantime and WR 6026, and for evaluating the efficacy of liposome-encapsulated drugs against cutaneous leishmanial infections.

A pilot investigation to evaluate the use of this animal model for the screening of candidate prophylactic leishmanial drugs has been completed. Results indicate that a regimen of Glucantime equivalent to that used clinically in man, i.e., 100 mg/kg/day x 10 days, when administered three days post inoculation with 5×10^6 amastigotes of L. mexicana, delayed the development of grossly visible lesions for seven weeks (see Figure 1).

Using this data as a basis for comparison, a collaborative protocol for the investigation of candidate prophylactic antileishmanial drugs has been initiated with LT CMDR Erich Stafford, Ph.D., Assistant Professor of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD.

III. USAMRDC CONTRACTED LEISHMANIAL PROJECTS.

The Leishmania Section, Division of Experimental Therapeutics, has been designated by USAMRD Command as monitor and technical advisor for a group of U.S. Army contracts for research on leishmaniasis. This research includes studies to characterize the antileishmanial activity of chemical compounds synthesized and/or provided by WRAIR and studies to evaluate the utility of new treatment techniques based on novel concepts of drug delivery.

Results of these contract studies have identified several promising chemotherapeutic leads which are being investigated.

IV. LIPOSOME-ENCAPSULATED DRUGS

The efficacy of antimonial drugs against experimentally induced visceral leishmaniasis in the hamster has been dramatically enhanced by incorporating these drugs into artifically produced multilamellar lipid spherules called liposomes. When liposomes of appropriate size and phospholipid composition containing Pentostam or Glucantime were administered intravenously, they were rapidly phagocytized by cells of the reticuloendothelial system, the cells which harbor the leishmanial organisms in an established infection. This allows gradual release of the active drug within the cells containing the parasite. The liposome encapsulated antimonials suppressed leishmanial infections in the hamster at doses of antimony less than 0.15% of that required for the unencapsulated drug, and the encapsulated drugs were equally effective against both early (3 day) and established (17 day) infections. Additional preclinical investigations are in progress.

A contract that provides the Army with the means of screening candidate compounds for activity against visceral leishmaniasis in the hamster model has revealed a series of related compounds with a high degree of antileishmanial activity.

The characteristics of the activity of this newly discovered series of 6-methoxy-4-methyl-8-aminoquinolines have been studied in the hamster model. The most active compound in this class (WR 6026) was approximately 700 times as active as the standard antimonial drug, Glucantime, against L. donovani when administered orally to hamsters. This compound was also approximately 700 times as active as Glucantime in vitro against axenic amastigotes of a mucocutaneous strain of L. braziliensis. Further preclinical efficacy and toxicological studies are in progress.

V. CRYOBANK PROJECT.

The collection of reference strains and new isolates of Leishmania has continued. Currently this collection consists of 288 specimens including 79 new strains and 91 new stabilates which were added this year. Fifty-four of these are primary human isolates which were made by this laboratory. Acquired stabilates of particular interest are a series of isolates of cases of canine visceral leishmaniasis from Spain, France and Algeria, and clinically documented Pentostam-resistant human visceral and cutaneous strains from Kenya.

VI. DIAGNOSTIC LABORATORY SERVICES.

As the result of an EPICON conducted in 1977 in response to a request for aid in the diagnosis of suspect cases of cutaneous leishmaniasis among personnel of the 82nd Airborne Division, Ft. Bragg, NC, a collaborative effort between the HCS, Ft. Bragg, and WRAIR was undertaken. The objective of this study was to determine the overall attack rate of cutaneous leishmaniasis among personnel exposed during 2½ weeks of training at the U.S. Army Jungle Warfare Center, Ft. Sherman, Canal Zone. A battalion of approximately 630 men was chosen. Prior to their scheduled departure for Panama, these men were bled for serum and were asked to fill out a questionnaire concerning their previous travel histories in leishmania-endemic areas. Approximately two months after the completion of jungle training the men were again bled and examined for persistent skin lesions. Thirty patients with suspect papular or ulcerative skin lesions were cultured by the needle aspirate-Schneider's medium technique. Most lesions were on the appendages below the elbow or knee with the majority on the lower leg. Leishmanial promastigotes were cultured from 11 of these servicemen in the endemic area. Approximately 3½ months after the return of this battalion to the U.S. a third serum sample was collected.

Sera collected on these individuals was submitted for seriological test. The indirect fluorescent antibody test conducted at WRAIR and by Dr. Bryce Walton at Gorgas Memorial Laboratory, the direct agglutination test conducted at CDC, Atlanta, GA, and the ELISA conducted by LAIR all failed to detect antibody against Leishmania in the sera collected at the time the positive cultures were acquired. To date the results of this study indicate an approximate attack rate of 16/1000. There were no differences in attack rates attributable to age or race correlation.

A similar study involving approximately 60 West Point Cadets who participated in jungle warfare training during August of 1978 is being conducted.

Because of this increased awareness of the occurrence of cutaneous leishmaniasis in U.S. military personnel, this laboratory has been involved in a service-wide evaluation of 75 suspected leishmania patients this year. From these, 29 primary human isolates have been obtained. During evaluation of patients with the needle aspiration and culture diagnostic technique, Schneider's insect medium + 30% v/v FCS was compared with the widely used NNN blood agar media. Schneider's was approximately

5 times more sensitive as a diagnostic tool for both Old and New World cutaneous leishmaniasis. In 55 patients from which cultures were attempted, promastigotes were observed in 38 of the Schneider's medium cultures in an average of 6.0 days, compared to only eight of the NNN cultures becoming positive in an average of 11.6 days (see Figure 2).

VII. CLINICAL TRIALS OF NEW PENTOSTAM TREATMENT REGIMENS.

Military patients returning to the U.S. from Panama or Brazil with cutaneous lesions of L. braziliensis of 4-12 months duration have often failed to respond to two or more courses of Pentostam therapy using the approved CDC regimen. Two revised Pentostam regimens, designed in an attempt to sustain more uniform blood levels of antimony, are being investigated under a WRAIR IND protocol. Initial results indicate the importance of early recognition and treatment of cutaneous lesions, as all of the first 11 cases with lesions of less than three months duration have been apparently cured with a single course of therapy with either the standard or experimental regimens. Blood levels of antimony in patients receiving the various regimens are being compared to determine the optimal course of therapy.

A compilation of data derived from military Leishmaniasis patients seen at WRAIR and treated at WRAMC during the 18 months period from April 1977 to September 1978 provides the following statistics: (1) 35 suspect Leishmania cases have accumulated 1468 man-days away from their units while under evaluation or treatment, a total of 4.02 lost man-years and an average of 41.9 man-days lost per patient; (2) 26 confirmed cases have accumulated 1409 lost man-days during evaluation or treatment, an average of 54.9 man-days lost per patient.

(3) The nine suspect cases that ultimately proved to be negative for Leishmania based on culture evaluation lost a total of 59 man-days away from their units or an average of 6.5 man-days lost per man.

(4) Based upon the actual pay grade of the individual patients involved and assuming that they were in the middle pay grade increments for their particular grade, the following figures were calculated: total cost of wages of all suspect cases of leishmaniasis was $38,115.00; wages paid to patients with confirmed leishmaniasis while under evaluation or treatment was $35,890.00; and wages paid to non-confirmed cases while under evaluation was $2,226.00. Total hospital cost incurred by the

Army for the treatment of the 26 cases of cutaneous leishmaniasis treated at WRAMC was approximately $139,400.00.

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 099 Chemotherapy and Chemoprophylaxis of Leishmaniasis

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a. Published:

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2. Alving, C.R., Steck, E.A., Chapman, W.J., Jr., Waits, B.V., Hendricks, L.D., Swartz, G.M., and Hanson, W.L.: Therapy of leishmaniasis: Superior efficacies of liposome-encapsulated drugs. Proc. Natl. Acad. Sci. 75(6):2959-2963, 1978.

3. Hendricks, L.D.: An in vitro drug screening system for Leishmania using axenically derived amastigotes. Published as a short communication in the abstracts of the Fourth International Congress of Parasitology, 19-26 August 1978, Warsaw, Poland, Section D-6, pg 87.

4. Hanson, W.L., Chapman, W.J., Jr., Kinnamon, K.E., Loizeaux, P.S., Steck, E.A., Davidson, D.E., and Hendricks, L.D.: Evaluation of the antileishmania activity of selected compounds against Leishmania donovani in the Golden Hamster. Published as a short communication in the abstracts of the Fourth International Congress of Parasitology, 19-26 August 1978, Warsaw, Poland, Section D-6, pg 88.

5. Alving, C.R., Steck, E.A., Chapman, W.L., Jr., Waits, V.B., Hendricks, L.D., and Hanson, W.L.: Liposome-encapsulated drugs: New therapeutic advance in leishmaniasis. Abstract published in the program of the American Society of Biological Chemists and The American Association of Immunologists, June 4-8, 1978, Atlanta, Georgia, Section 219: Bacterial and Parasitic Infections, B-81 #3164.

6. Steck, E.A., Alving, C.R., Chapman, W.L., Jr., Waits, V.B., Hendricks, L.D., Swartz, G.M., Jr., and Hanson, W.L.: Experimental visceral leishmaniasis: Improved therapy by use of liposome-encapsulated drugs. Abstract published in the program of the 53rd annual meeting of the American Society of Parasitologists, 5-10 November 1978. Chicago, IL, Abstract 142, pg 75.

b. In Press:

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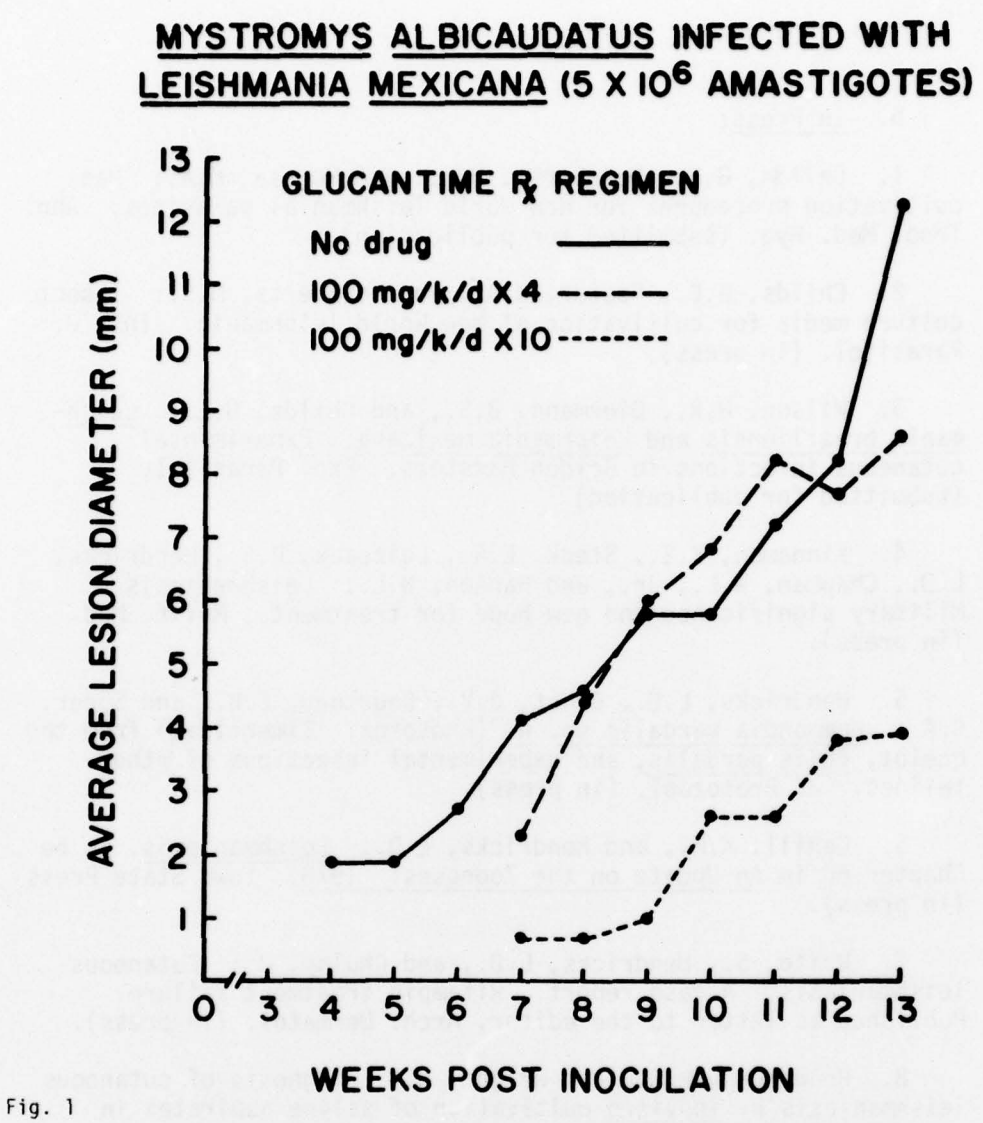


Fig. 1

Leishmania mexicana lesion development in Mystromys albicaudatus treated with various regimens of Glucantime administered three days after an infection of 5 x 10^6 amastigotes.

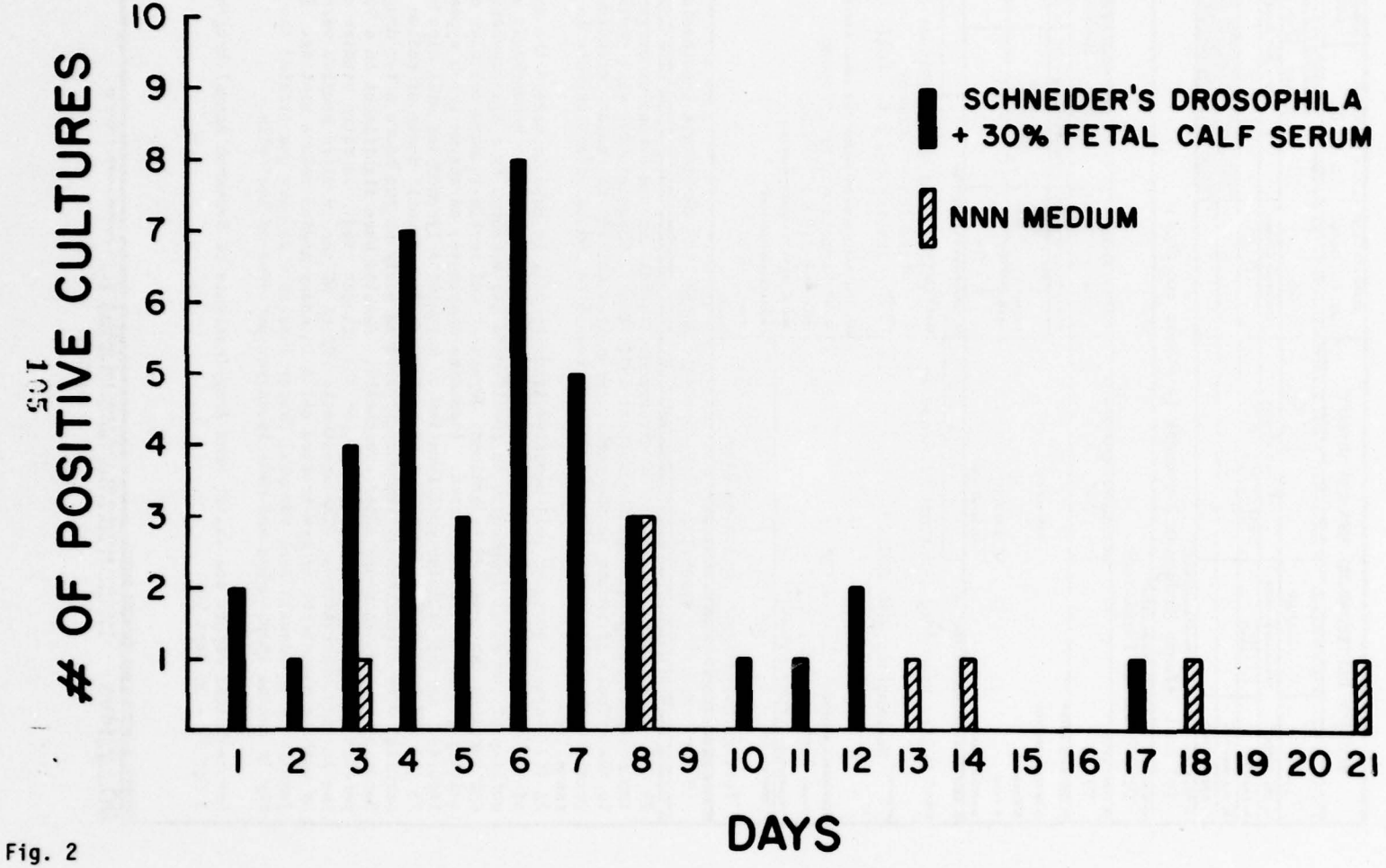


Fig. 2

Comparison between the initial detection of leishmanial promastigotes in Schneider's Drosophilia Medium and NNN Medium cultured from needle aspirates of human cutaneous lesions.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0C 6462	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A 91C	00	100
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*
(U) Host Defense Mechanism Responses to Stress and Shock

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1	70
	CURRENT 79	1	80

19. RESPONSIBLE DOD ORGANIZATION
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TELEPHONE: (202) 576-2673
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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Cell-mediated immunity; (U) Hemorrhagic Shock; (U) Barbituate Anesthesia

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To evaluate the effect of experimental shock and the stress of surgery on the immunological competence of the subject individual. These studies will provide insight to the effects of trauma on the combat casualties ability to resist infection. It is possible that therapeutic approaches will result to reduce infection rate in combat casualties.

24 (U) Various tests of cell-mediated immunity will be used to assess the competence of the immune system. Using canines subjected to anesthesia or hemorrhagic shock, the ability of the small lymphocyte to proliferate (as measured by a blastogenesis assay) and function (as measured by antibody dependent and lectin-induced cellular cytotoxicity tests) will be monitored. Adenosine deaminase, an enzyme which appears to be important in the cellular proliferation of stimulated lymphocytes will also be monitored.

25 (U) 77 10 - 78 09 Seven canines administered anesthetic doses of sodium pentobarbital were significantly immunosuppressed as early as two hours after drug injection. Recovery started 24 hours after anesthesia. Results were significant to a 95 to 98 percent confidence level depending upon the mitogen used. In vitro studies confirmed the in vivo observations. The anesthetic effect of the in vitro studies were reversed by washing pentobarbital pre-treated cells in assay medium before plating. In conjunction with Roswell Park Memorial Cancer Institute we have quantitated the ADA activity in canine lymphocytes and made isoenzyme patterns of the cells.

For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 100 Host defense mechanism responses to stress and shock

Investigator
Principal: MAJ Joseph Formeister

The effect of anesthesia on cell-mediated immunity with a canine model.

A. Background and Statement of the Problem. Infection is a leading cause of morbidity and mortality following anesthesia and surgical insult or trauma. It is not known to what extent cell-mediated immunity is compromised in these states, and hence responsible for permitting bacterial invasion to go unchecked.

Several investigators have suggested that barbituates used during the induction of clinical anesthesia severely compromise the ability of the small lymphocyte to proliferate. Lymphocyte proliferation would be important if a proper immune response is to be generated against invading pathogens. The studies reported in the literature are complicated by multiple drug administrations, the nature of the patients underlying disease, assay techniques, etc. Furthermore, there are no studies on the functional capability of the circulating lymphocytes.

There are also scattered reports which suggest that the proliferation of lymphocytes from traumatized patients and individuals with sepsis is diminished. All of the problems sited above, which render interpretation of the anesthetic effects on immune responsiveness impossible, are also applicable to these reports as well.

The purpose of this study is threefold: First, to develop a canine model for the study of cell-mediated immune responses. Second, to evaluate the effect of anesthetic agents on cellular immunity responses using this model system and third, to study the effect of hemorrhagic shock on cellular immunity responses using this same model system.

B. Experimental Approach. Our work to date deals with the effect of barbiturate anesthesia on the proliferative capacity of the small lymphocyte.

Lymphocytes separated from whole blood samples collected at intervals from 5 healthy canines before, during, and after intravenous administration of an anesthetic dose of pentobarbital were washed three times in culture medium before final plating. Prepared cells were incubated for 3 days in approporiate concentrations of fractionated phytohemagglutinin P, pulsed with ^{3}H thymidine, harvested, and counts per minute (c.p.m.) recorded. Lymphocytes for in vitro testing were separated from whole blood samples of 6 healthy previously unanesthetized canines and each sample treated in 2 ways. In experimental group I, lymphocytes were incubated for 3 days with mitogen in anesthetic (1.5 - 3.0 mg %) or toxic (6.0 mg %) pentobarbital concentrations before H-thymidine pulsing. In experimental group II, lymphocytes were pretreated for 3 hours in the aforementioned barbiturate concentrations, washed 3 times in culture medium and then incubated for 3 days with mitogen.

C. Results and Discussion. In vivo testing showed a significant decrease in ^{3}H thymidine uptake in cultures of lymphocytes sampled 3 hours after pentobarbital injection when compared with preanesthetic control cultures ($p<0.05$). Full recovery was appreciated in cultures from cells sampled at 24 hours, and was coincident with disappearance of pentobarbital from the blood stream. In vitro lymphocyte cultures (experimental group I) showed significantly diminished ^{3}H thymidine uptake when compared with cultures incubated in the absence of barbiturate ($p <0.05$). In experimental group II, washing pentobarbiral pretreated cells with culture medium reversed the drug's inhibitory effect.

In conclusion, pentobarbital anesthesia can significantly depress the immune response as monitored by a mitogen-induced blastogenesis assay, using canine lymphocytes both in vivo and in vitro. This suggests that barbiturates administered during the induction phase of clinical anesthesia may contribute to the diminished immune response reported after surgery. Furthermore, whereas the proliferative ability of lymphocytes pretreated with pentobarbital in vitro can be reversed by washing the cells in culture medium, lymphocytes exposed to pentobarbital in vivo are so altered that washing alone does not restore their ability to proliferate normally.

D. Recommendations. Pentobarbital anesthesia can significantly inhibit mitogen induced blastogenesis or the ability of lymphocytes to proliferate. Our next attempt will be to evaluate the in vivo and in vitro effect of sodium pentobarbital on the functional capacity of the circulating lymphocyte.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 100 Host defense mechanism responses to stress and shock

Literature Cited.

Publications:

1. Formeister, Joseph F., Sako, K., Razack, M.S., and Aungst, W.S. Myxdema coma and inappropriate antiduretic hormone secretion after deep neck irradiation, J. Surg. Oncology. In press.

2. Formeister, J.F., and Tritsch, G.L. Adenosine deaminase levels in construction workers with asbestos contact dermatitis. J. of Medicine. In press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6463	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A 91C	00	101
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Thrombotic Mechanisms in Stress

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

01622 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:

b. NUMBER:

c. TYPE: — d. AMOUNT:

e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1	99
FISCAL YEAR CURRENT 79	1	86

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Platelet Survival; (U) Thrombotic Events; (U) Platelet Inhibitors; (U) Vascular Trauma

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Platelet activity and survival will be evaluated in an animal model of surgical stress. The mechanism of action of various antithrombotic agents will be determined. The role of platelets and thrombotic mechanisms in wound healing will be evaluated. These studies are militarily relevant in terms of potential value in treating arterial and venous thrombosis secondary to peripheral vascular trauma.

24 (U) Rabbits and dogs will be used to devise a model with shortened platelet survival. Platelet function and thrombogenesis will be assessed in conditions under which platelet survival is shortened. A comparison of in vivo and in vitro effects of drugs inhibiting platelet function will be studied using platelet aggregometry, malonyldialdehyde production by platelets, and platelet ^{14}C-serotonin release. Wound healing strength in rabbits will be studied under conditions of severe thrombocytopenia broughtabout by injecting sheep anti-rabbit platelet serum.

25 (U) 77 10 - 78 09 It was found that shortening platelet survival time results in increased sensitivity of platelets to aggregating agents, in vivo release of serotonin, and impaired platelet thrombogenesis. Aspirin, in all dosages tested, was found to markedly suppress in vitro platelet function and there was no dose-response relationship. The impairment of in vivo platelet thrombogenesis, however, was found to be highly correlated with the aspirin dose. Preliminary results suggest that severe thrombocytopenia impairs wound tension strength.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 101 Thrombotic Mechanisms in Stress

Investigators
Principal: MAJ G. Patrick Clagett

I. In vivo interactions between platelets and prosthetic arterial surface

A. Background and Statement of the Problem. It is widely accepted that platelets adhere and aggregate on prosthetic surfaces in the arterial circulation and that this interaction may result in thromboembolism. The purpose of the present investigation has been to find out if there are changes in platelet function resulting from this interaction. The implication is that if there are changes in platelets and their function, then this would influence subsequent thrombotic and hemostatic events.

B. Experimental Approach. Thoracoabdominal aortic bypass grafts of woven Dacron were placed in 15 dogs to create an animal model in which the arterial blood would be continuously and chronically exposed to a prosthetic surface. Biochemical, morphological, and functional studies of platelets were carried before and after placement of the grafts. To divorce the influence of major surgery alone upon the platelet studies, 8 sham animals undergoing thoracotomy without insertion of graft were studied in identical fashion. In all of these animals, the following specific studies were carried out. Platelet survival time was measured using ^{51}Cr labelled autologous platelets. Dose response platelet aggregometry was carried out to determine the lowest or threshold dose of adenosine diphosphate (ADP) or collagen necessary to bring about full, sustained, and maximal platelet aggregation. Biochemical measurements of platelet serotonin, adenosine diphosphate, and adenosine triphosphate were also carried out. Studies of platelet uptake and release of ^{14}C-serotonin were also performed. Morphologic studies include platelet electron microscopy and platelet size determination using the Coulter Counter electronic particle size analyser. Additionally, platelet prostaglandin synthesis was assessed by measuring thrombin stimulated malonyldialdehyde production, a prostaglandin endoperoxide metabolite. In addition to the platelet studies, several routine hematologic and coagulation tests were performed including serial determinations of hematocrit, white cell count, platelet count,

prothrombin time, thrombin time, partial thromboplastin time, euglobulin lysis and fibrinogen levels.

C. Results and Discussion. In animals with grafts the platelet count significantly decreased and returned to normal levels 6-8 weeks after graft placement. In those undergoing sham operations, thrombocytosis occurred as the expected response to major surgery. This suggested that in animals with grafts, platelets were being consumed.

Consumption of platelets was confirmed by platelet survival studies. In animals with grafts platelet survival time was significantly reduced when compared to pre-op measurements. In animals undergoing sham operation, platelet survival time was unchanged.

In the animals with grafts the sensitivity to aggregating agents was markedly increased in that the threshold aggregating dose of ADP was reduced to 1/7 the pre-op level and that of collagen to 1/2. In the sham operated animals there were no changed in these parameters.

Platelet serotonin levels in graft animals were significantly reduced for up to 4 months after placement of the prosthetic surface. During this time, serotonin levels gradually returned to normal. Also during this time platelet survival time lengthened although it did not return to normal. These data suggested that platelet interaction with the graft led to platelet release of serotonin and that as the interaction lessened, platelets retained more of their serotonin. In sham-operated animals, there were no changes in platelet serotonin levels.

At present we can document no changes in platelet adenin nuclotide levels (ADP and ATP). This is still under investigation, however.

The platelet sizing studies have shown no significant changes in mean cell volume and the electron microscopic studies are incomplete. Also incomplete but still under investigation are the prostaglandin studies.

D. Conclusions and Recommendations. These studies have documented that platelet interaction with prosthetic arterial surfaces results in marked changes in platelet function and biochemistry. These findings will be presented on the Surgical Forum at the American College of Surgeons meeting in October 1978. The next phase of this project is to find out the relevance of

these platelet changes ot thrombogenesis.

II. Platelet Changes Induced by Prosthetic Arterial Surfaces and Platelet Thrombogenesis.

A. Background and Statement of the Problem. In the first project, multiple changes in platelets and platelet function occurred with exposure to prosthetic arterial surfaces. The purpose of this project was to determine the influence of these changes on subsequent platelet thrombogenesis.

B. Experimental Approach. To address this problem, it was necessary to create a reproducible animal model of thrombosis dependent upon platelet functions. Arteriovenous shunts using silastic tubing were placed in dogs in the femoral artery and veins or the internal carotid artery and external jugular vein. Flow rate within the shunt was controlled at 200 ml/min using an adjustable partially occluding clamp on the tubing. After stabilizing the flow-rate, the tubing was clamped and a piece of 40 u polyester mesh was interposed into the tubing in the manner of a diaphragm. The clamps were removed, flow re-established, and the time for flow to cease was recorded. Cessation of flow occurred because of the formation of an occlusive thrombus that formed on the polyester mesh. Experimental results detailed below documented that the formation of the thrombus and occlusion time (T-O) was dependent upon platelet function.

This experiment was carried out pre-operatively and 2-6 months post-operatively in many of the dogs undergoing graft and sham operations mentioned in the first project.

C. Results and Discussion. Multiple experiments determined that the A-V shunt T-O was dependent upon platelet function. First, the T-O was strikingly inversely correlated with the flow rate. That is, as the flow rate was increased the occlusion time decreased. This suggested that T-O was dependent upon platelet function because as the flow rate increased more platelets were delivered to the mesh and more platelet-mesh and platelet-platelet interactions occurred. Such flow dependent phenomena is classically descriptive of platelet participation in thrombogenesis.

Further, inhibitors of platelet function would reproducibly prolong A-V shunt T-O. Most striking in this regard, were aspirin and prostaglandin E_1, both of which markedly prolonged T-O.

Lastly, the occlusive thrombi forming upon the mesh were examined by light and electron microscopy. These thrombi were almost entirely composed of degranulated platelets.

In animals with grafts in place, the A-V shunt T-O was prolonged 2-3 fold over pre-operative values. This striking change was not observed in animals undergoing sham operation.

D. Conclusion and Recommendations. These findings suggested that platelet changes brought about by interaction with prosthetic arterial surfaces resulted in imparied platelet participation in thrombogenesis. These results will be presented at the Third National Conference on thrombosis at the annual American Heart Association meeting in Dallas, Texas in November 1978.

III. Urinary Excretion of 5-hydroxy Indole Acetic Acid (5HIAA) as an Index of Platelet Survival Time

A. Background and Statement of the Problem. The measurement of platelet survival time has clinical relevance in that it may predict patients predisposed to thrombosis as well as identify those who will benefit from platelet inhibitor therapy. Among the many isotopic methods of labelling platelets for measuring platelet survival, the most widely used is the ^{51}chromium technic. This technic, although highly accurate, is time consuming, cumbersome, and requires special skills which have retarded its widespread clinical application. Other non-isotopic technics have recently been introduced but have been found, in general, to be unsatisfactory.

It is proposed that the urinary excretion of 5-HIAA might reflect alterations in platelet survival and provide a simple means of its determination. The rationale for this is that platelets release serotonin during aggregation. In vivo, the platelet release of serotonin has been suggested by increased urinary excretion of 5-HIAA, a serotonin metabolite. This has been noted inthe postoperative state when platelet survival is shortened. It has also been noted that inhibitors of the platelet release reaction such as aspirin and dextran significantly decrease the post-operative rise in 5-HIAA. These considerations have led us to formulate the hypothesis that changes in platelet survival will be reflected in alterations in urinary excretion of 5-HIAA.

B. Experimental. Rabbits were initially used to devise an animal model of shortened platelet survival time and this was discussed in detail in last years annual report, Section I. This model was found to be unsatisfactory because of difficulties in accurate urine collections. The model was abandoned and the dogs with thoracoabdominal aortic grafts were used since they had documented shortened platelet survival time. Measurements of 5HIAA excretion, platelet serotonin levels, ^{14}C serotonin uptake and release uptake and release and platelet survival time were carried over a 6 month period before and a 5 month period after placement of grafts. At different intervals during these periods the animals were treated with aspirin, aspirin plus dipyridamole, and sulfinpyrazone and the measurements repeated.

C. Results and Discussion. These studies are still in progress but preliminary data indicate that as platelet survival is shortened after graft placement, platelet serotnin levels drop, and urinary 5HIAA excretion increases. However, treatment with platelet inhibitors seem to have no influence on these parameters. That is, with grafts in place, treatment of the animals with platelet inhibitors does not affect the shortened platelet survival time, reduced platelet serotonin levels, or urinary 5HIAA excretion.

D. Conclusions and Recommendations. The increase in urinary 5HIAA excretion with shortening platelet survival time is of interest. However, the inability of platelet inhibitors to alter this and other parameters is disappointing. It is unclear, at present, whether measuring urine 5HIAA and/or platelet serotonin will be of any value in determining states of altered platelet kinetics.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 101 Thrombotic Mechanisms in Stress

Literature Cited

Publications:

1. Clagett, G.P., and Collins, G.J.: Platelets, Thromboembolism and the Clinical Utility of Antiplatelet Drugs. Surgery, Gynecology and Obstetrics, Aug. 1978

2. Clagett, G.P., and Olsen, W.R.: Non-mechanical Hemorrhage in Severe Liver Injury. Annals of Surgery, April 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6464	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. [illegible]

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	102
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Chemotherapy and Chemoprophylaxis of African Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	Cont.	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.8	53
FISCAL YEAR CURRENT 79	0.8	84

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Trypanosomiasis; (U) Drug Development; (U) Biology; (U) Chemistry; (U) Toxicology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against African trypanosomiasis, a debilitating and frequently fatal disease which would pose a serious threat to military personnel operating in Central Africa. Epidemic transmission during military operations is a distinct possibility. Currently available drugs are neither safe to use nor fully effective in therapy. There are no adequate drugs for prophylaxis.

24. (U) Laboratory models developed in this laboratory will be utilized to test selected chemical compounds from the malaria drug inventory for activity against Trypanosoma rhodesiense: (1) in vitro, (2) in laboratory mice, (3) in cynomolgus monkeys.

25. (U) 77 10-78 09. The conditions for incorporation of radiolabelled thymidine and glucosamine in vitro by blood from trypomastigotes have been optimized. Dose response curves for both indicators have been demonstrated with a variety of effective drugs. A major problem continues to be the uncertainty of the glucosamine label specificity for the glycoprotein coat. To provide a useful screen, therefore, leucine will be used in place of glucosamine, though it is not a specific label for the glycoprotein coat. Optimum conditions for the simultaneous incorporation of thymidine and leucine are now being evaluated. In addition, work will continue toward the development of a relatively specific glycoprotein coat label. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78.

*Available to contractors upon originator's approval

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PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 102 Chemotherapy and Chemoprophylaxis of African Trypanosomiasis

Investigators
Principal: LTC David E. Davidson, Jr., VC
Associate: Gerald J. McCormick, Ph.D.
MAJ Robert E. Desjardins, MC

1. Description

African trypanosomiasis is a parasitic disease which poses a potentially serious threat to U.S. military personnel who may be required to operate in the large areas of Africa in which this disease is endemic. All drugs which are currently available for use against this disease are less than fully effective and/or have serious side effects. Efforts to develop drugs effective against this disease include screening and evaluation of candidate chemotherapeutic and chemoprophylactic agents by in vivo and in vitro techniques and elucidation of metabolic processes of the trypanosomes by laboratory investigation.

In-house research is supported by and coordinated with contract research monitored within the Department of Parasitology, Division of Experimental Therapectics, WRAIR

2. Progress

a. Primary Mouse Screen

The primary in vivo screen in mice utilizes the Wellcome CT strain of Trypanosoma rhodesiense. Compounds are administered subcutaneously to mice which have received a lethal dose of parasites (intraperitoneal injection of 0.5 ml of a 1:50,000 dilution of heparinized blood from heavily infected donor mice). Active compounds extend survival time beyond that of untreated infected control mice (4.5 $\pm$ 0.3 days). Treated mice surviving 30 days are considered to be cured.

Since 1972, approximately 14,000 compounds have been screened and nearly 400 have had reportable activity. In the past year 3000 of these compounds were tested and activity was exhibited by

123 compounds. The greatest number of "actives" (37) were in the triphenyl phosphonium series. There were also 16 novel arsenicals with activity.

b. Induction of Drug Resistance in Trypanosoma Rhodesiense

Mice infected with Wellcome CT strain Trypanosoma rhodesiense were treated orally with suboptimal doses of antitrypanosomal drugs in an attempt to induce drug resistant strains.

ICR/HA mice were inoculated intraperitoneally with infected blood on day 0, and treated orally or subcutaneously with the test drug on day 0 and day 1. On day 3 or 4, the blood of these mice, containing parasites, was inoculated into a second group for further drug pressure.

A drug sensitive strain of T. rhodesiense was made progressively more resistant to Berenil over a period of 2 months of drug pressure through 22 line passages. The strain was 520-fold resistant to Berenil at that point. After 6 months and 61 passages under continued drug pressure, resistance to Berenil has not increased further.

The activity of other antitrypanosomal drugs against the Berenil resistant line was determined. The resistant line was fully sensitive to Suramin, but was highly cross-resistant to Stilbamidine, Melarsoprol, and Pentamidine.

The Wellcome CT strain was also exposed to drug pressure with Suramin, Melarsoprol and Stilbamidine. Suramin-induced resistance has continued to increase progressively through 14 months of drug pressure and 136 line passages. Melarsoprol resistance developed relatively rapidly in 6 weeks through 16 line passages under Melarsoprol pressure, reaching 64-fold by the 16th passage. Stilbamidine resistance emerged slowly, reaching 32-fold after 14 months and 138 line passages under Stilbamidine pressure. The Stilbamidine resistant line is also cross-resistant to Berenil and Melarsoprol.

c. In Vitro Antitrypanosomal Drug Screening System

During the reporting period, this laboratory has continued to develop an automated in vitro system for evaluation of potential antitrypanosomal compounds. Several changes have been introduced requiring further optimization of culture conditions. The system is now functional and should soon be producing weekly

reports in a standard format.

The parasites, T. rhodesiense, are grown in Walter Reed rats and harvested for the culture system as outlined in previous reports. Approximately ten rats per week will be used to maintain the system. The parasite is maintained by continuous passage in vivo and stabilates are maintained in liquid nitrogen storage.

Previous efforts to obtain reliable baseline data for the incorporation of ^{14}C-glucosamine as a glycoprotein coat label were not successful. The avid utilization of glucose by blood stream forms of the parasite is the major obstacle to successful use of this interesting label. Several modifications of the culture media were investigated to improve the uptake of ^{14}C-glucosamine with varying results. Alternative nutrient energy sources, such as glycerol and glycerol 3-phosphate, were also evaluated. None of these alterations provided the reliable results needed for a general screen.

A decision was made recently to use a labelled amino acid instead of an amino sugar in conjunction with a DNA label and this has proven to be quite satisfactory. The system now employs a dual label with ^{14}C-leucine and ^{3}H-thymidine as indicators of drug activity. The parasites are cultured in RPMI 1640 with 5 gm/L of glucose and a bicarbonate buffer system. Antibiotics are not used in the culture system. Optimum conditions with respect to the concentration of thymidine, leucine, and pH in the present medium have been established, and the kinetics of incorporation of both isotopes have been characterized.

The ability to detect a differential effect on incorporation of each of the labelled precursors has been demonstrated using thioguanine to inhibit ^{3}H-thymidine and puromycin to inhibit ^{14}C-leucine incorporation. Other compounds with less specific antitrypanosomal activity tend to affect both parameters of viability.

The data analysis programs for the Tektronix 4051 Graphics Computing System have been completed. Data recorded on paper punch tape is now read automatically into the computer. A log-transformed pseudo-linear regression is performed on the data for each compound, generating an estimate of the ED-50, 95% confidence intervals, and statistical analysis by ANOVA of the regression. There are two ED-50 values for each compound, representing 50% inhibition of uptake for each of the two labelled precursors.

A number of known effective antitrypanosomal drugs have already been evaluated in this system. Most of these have shown a significant effect *in vitro*. Examples of 4 compounds indicating their ED_{50} values are presented in Table 1. Astiban is used as a positive control because of its potency and reproducibility. Pentamidine and ethidium bromide are also very effective in this system. Interestingly, the complex compound, Suramin, is quite ineffective *in vitro*. This may be due to any of several possible factors. The drug may require *in vivo* metabolism to an active form, or its mechanism of action may be such that we are unable to detect activity in a three hour culture, with the two precursors employed in the system. Suramin is known to be a slow acting drug *in vivo*.

Future work to establish the value of this system as a primary screen is planned. A number of correlative *in vitro* and *in vivo* studies with several classes of compounds are anticipated. Incorporation of the labelled precursors as an index of parasite viability is also being investigated.

In addition to providing a primary screening function for the antitrypanosomal drug development program, this *in vitro* system will be employed to study mechanisms of drug action, drug interaction and drug resistance phenomena. A number of specific drug resistant lines of *T. rhodesiense* are now available. These will be characterized and evaluated for cross resistance *in vitro*.

Table 1

Drug Induced Suppression of Incorporation of ^{3}H-Thymidine and ^{14}C-Leucine by Trypanosoma rhodesiense in Culture.

Compound	Incorporation of ^{3}H-Thymidine		Incorporation of ^{14}C-Leucine	
	ED_{50}*	95% Confidence Limits	ED_{50}*	95% Confidence Limits
WR 7035 Sodium Stibocaptate (Astiban^{R})	1.57	1.24-1.99	0.30	0.12-0.77
WR 141377 Ethidium Bromide	0.59	0.49-072	0.48	0.37-0.63
WR 173491 Suramin Sodium	>12.5	-	>12.5	-
WR 4931 Pentamidine Isethionate	0.76	0.54-1.07	0.62	0.38-1.00

*Concentration (µg/ml) of test compound to suppress incorporation of label by 50%.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	103
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Infections of Cultured Intestines by Pathogenic Microbes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	Cont	DA	C In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.5	39
FISCAL YEAR CURRENT 79	1.5	30

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ASSOCIATE INVESTIGATORS

NAME: CHO, HAN, Ph.D.

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Organ Culture; (U) Intestine; (U) Phase Microscopy; (U) Electron Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To clarify interactions between cultured gut mucosa and pathogenic microbes including bacteria, viruses and microbial toxins. The results will provide new information which should clarify the pathogenesis of acute diarrheal diseases in military personnel.

24(U) Conventional morphologic techniques including phase contrast, light and electron microscopy, histochemistry, immunochemical, biochemical, and isotope tracer methods are being used. Methods such as interference microscopy and cinematography will also be added as the work progresses.

25(U) 77 10-78 09 Utilizing a modified Rose's tissue culture chamber and conventional Falcon culture bottles, the viability and growth of the guts of the guinea pigs and mice in vitro have been continuously studied. Fetal small and large intestine epithelial cells (absorptive, goblet, Paneth and Argentaffine cells), endothelial cells, mesenchymal cells (lymphocytes, macrophages, mast cells, fibroblasts, smooth muscle and nerve cells) could be grown and remain viable up to two weeks while adult counterparts do not survive more than 2 days. Current efforts have been directed toward the synthesis of DNA, glycoprotein, cyclic AMP and immunoglobulin A. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 103 Infections of Cultured Intestines by Pathogenic Microbes

Investigators:
Principal: Akio Takeuchi, M.D.
Associate: Han Cho, Ph.D.

Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured intestine will be employed in: (1) Studies of responses of the intestinal mucosa and submucosa to various microbes and microbe-derived toxins; special attention will be paid to cinematographic recordings as well as conventional static observations on various cellular interactions of the intestinal epithelial mucosa with various microbes and toxins; (2) Studies of replication sites of certain enteroviruses in the cultured intestine.

These studies should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel.

Background:

Organ culture techniques have proven to be of considerable value in the study of respiratory tract infections in man and animals. Recently, interest has developed in the organ culture of enteric tissues for the cultivation of viruses (Dolin, 1970, Derbyshire and Collins, 1971, and Rubenstein et al., 1971) provided evidence of the multiplication of viruses in organ cultures of the small intestine of the human embryo. Dolin et al. (1972) reported that viral antigens from viruses which belong to different viral groups were successfully detected in human fetal intestinal organ cultures by immunofluorescent techniques. Kagnoff et al. (1972), in their organ culture study of adult rabbits, have reported that the metabolic function and synthesis of macromolecular substances including secretory IgA were still active after 24 hours culture in a Petri dish. Explants of human small intestine of normal subjects and patients with ulcerative colitis were maintained in culture up to 24 hours (Eastwood and Trier, 1973).

Most of the chambers used by the above investigators appeared adequate for the growth of certain viruses and for evaluation of limited metabolic activities of the cultured intestine. These chambers do not allow for an immediate and accurate sequential method of morphologic and physico-chemical evaluation of intestines growing in culture. This shortcoming prompted the development of a new chamber allowing a convenient observation during actual organ growth. The chamber we have developed by modifying Rose's chamber provides easier handling of tissues and better visualization of growing cells and tissues under the phase contrast microscope than conventional culture chambers.

Results:

I. Culture Chambers

A. Modification of Rose's Organ Culture Chamber: This revised system not only provides for easier handling of tissues than the old chamber, it also offers more precise visualization of growing cells and tissues under the microscope. By adding polyethylene gas exchange tubes to the old Rose's chamber model, the medium can be continually exposed to gases in the incubator for pH control. The previously used method of inserting dialysis strips to hold tissue fragments in the chamber was replaced in the revised method: collagen gels have been successfully extracted from rat-tail in our laboratory and are used to form films of collagen to which explants easily attach. In this new system, normal tissues display excellent survival, growth rates and the architecture of the tissues can be maintained to direct them toward normal maturation.

B. Falcon T-flask: Another organ culture chamber is also in use for culturing fetal intestine tissue. Survival and development of normal tissue can be observed under an inverted microscope for periods up to 3 weeks.

II. Culture Media

Several different media, i.e., Eagle's minimum essential medium (EDEM), Ham F-12, Basal medium Eagle, Medium-199, and Trowell's medium, were initially tested for their ability to support the growth of the explants. EDEM was selected for primary use because it gave the most consistent results in maintaining colonic explants in culture for extended periods of time when compared to the other media.

A. Development of a New Medium: Our present studies of media include special supplements of glucose and methionine. Cultures

in the early stages of growth rapidly consume great amounts of glucose. Methionine is a major amino acid component of mucoproteins. In addition, concentration of vitamins and hormones (specifically hydrocortisone, insulin, and thyroxine) in the selected medium are being monitored because of their effects on differentiation of intestinal cells in animals. Several types of sera have been examined for their ability to sustain explant survival, specifically horse, human, and fetal bovine serum (FBS) in progressive concentrations of 5-20%. So far the best results were obtained by using FBS (10%).

III. Phase Microscopic Observations on Cultured Intestines

A. Modified Rose's Chamber: Small fragments 1 mm in length of intestines from mouse embryo of mid-gestation (12-14 days) have been routinely cultured in modified Rose's chambers for 7 to 10 days and in a few cases up to 14 days. All cases had viable epithelium at 10 days. However, our latest data show an increase in survival time of the cultures, with viable epithelium appearing after 10 days in culture. When cylindrical pieces of intestine preserve their normal shape in culture, they contract rhythmically. Contractions commence only after a period of latency which probably corresponds to the time taken for the development of a muscular wall.

IV. Physico-chemical Characterization of Organ Cultures

Incorporation of precursors into cellular macromolecules, and immuno-tracers all served to initiate analyses of function and metabolism of intestinal tissues growing in modified Rose's chambers. A small percentage of Falcon chambers were also used in formulating these analyses.

A. Protein and Glycoprotein Synthesis: The explants were incubated with medium containing L-leucine [1-^{14}C] or D-glucosamine hydrochloride [1-^{14}C] for 4 to 12 hours. At one to three days intervals, the explants were homogenized in chilled 0.04% $CaCl_2$ and an aliquot of this homogenate was used to determine total protein or glycoprotein concentration using the Lowry method. Acid insoluble proteins were then precipitated from the remaining homogenate by addition of 30% trichloroacetic acid and 6% phosphotungstic acid. The pellet was washed with chloroform-methanol (1:1 by volume), dried, and then dissolved in 0.5 N NaOH for determinations of radioactivity. Analysis of secretory products of these explants was obtained by dialyzing their culture medium for 2 to 3 days against exchanges of 0.15 M NaCl and then measuring the radioactivity present in the dialyzed medium.

This dialyzed solution is being examined for the presence of IgA by immunodiffusion with rabbit anti-mouse IgA serum (Miles Lab.).

Conclusion and Recommendation:

1. One of the major difficulties encountered in culturing tissues in closed organ culture chambers is maintaining the proper level of pH for tissue growth; this problem was overcome by our addition of polyethylene gas exchange tubes to Rose's chamber systems. In this chamber, collagen-coated coverglass improves attachment of explants without using dialysis strips. In contrast to previous observations, we have been able to maintain mouse intestine in culture for up to 3 weeks with satisfactory preservation of mucosal morphology and continuation of epithelial cell proliferation.

2. Prolonged in vitro steady state protein and glycoprotein synthesis by intestinal explants have for the first time been demonstrated in our laboratory. Further investigations involving stimulation of mucosal adenyl cyclase, with subsequent elevation of cAMP level by bacterial enterotoxins associated with diarrhea will be initiated.

3. The formation of this epithelial layer in culture chambers offers a new, promising experimental model to study the mechanism by which epithelial elements repair mucosal injuries caused by enteric pathogens.

B. Deoxyribonucleic Acid (DNA) Synthesis: The explants were incubated with medium containing [methyl-^{3}H]-thymidine (1 Ci/ml, 617 Ci immole) for 4 to 12 hours and homogenized in 1 N NaOH. DNA was precipitated by addition of 1 N perchloric acid. The pellets were washed and suspended with 0.2 N perchloric acid. From this suspension DNA concentration was determined by the diphenylamine reaction for radioactivity.

C. Falcon Chambers: An interesting observation is frequently made in cultures growing in Falcon chambers. A flat layer of epithelial cells grows over the monolayer of fibroblast cells which are derived from the mesenchymal tissue of the intestinal explant. Cells at all levels of differentiation participate in the process. Mature columnar, Paneth, and goblet cells have been identified, as well as cells which were comparatively undifferentiated by PAS-positive staining technique. This flat epithelium might be formed by migration of the newly generated epithelial cells to the tip of the cylindrical intestinal fragment. This observation suggests that the epithelial cell has special requirements for attachment and growth and that the appropriate charge on the surface of mesenchymal tissue in vivo may be one of the requirements for normal epithelial growth and maturation. If fibroblast cells or mesenchymal tissue snythesize de novo a substance necessary for epithelial growth, the growth or maturation factor could be isolated.

Cultured explants of intestinal fragments from mouse embryos in early stages of gestation (10-12 days) develop new villi in vitro within 4 to 7 days. The newly formed villi maintain their shape in culture and continue differentiation. Explants obtained at later developmental stages of the fetuses do not reproduce intestinal villi. This phenomenon was often observed both in Falcon T-flask cultures and in the collagen-coated Rose's chamber systems.

D. Light and Electron Microscopy: The viability of the explant has also been monitored by observing the morphologic changes of the intestine using both light and electron microscopy.

Pending further investigations, the results obtained from the above experiments show that maximum levels for incorporation of radiolabelled leucine into protein and of radiolabelled glucosamine into glycoprotein were reached by the fifth or seventh day in culture. During the following 3 to 5 days the radiolabelled protein was steadily secreted into the culture medium. The highest level of DNA concentration was also reached at the fifth or seventh day in culture.

E. Secretory Immunoglobulin A (IgA) Secretion: The cultured medium was pooled, centrifued, dialyzed, and then concentrated tenfold by diaflo ultrafiltration using an XM-50 membrane (Amicon Corp.).

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6468	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	106
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

Computer simulation of red blood cell metabolism

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	2	65
FISCAL YEAR CURRENT 79	2	100

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Mr. John A. Prichard (GS-11) DA

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Metabolism; (U) Computer Simulation; (U) Blood Preservation

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To develop a computer simulation model of intermediary metabolism in the erythrocyte, based on data both in existence and to be obtained in our laboratories. By constructing separate computer models of the metabolism of uninfected erythrocytes, malaria-infected erythrocytes, and free malaria parasites, we can gain a better understanding of how the parasite utilizes the metabolic machinery of the host erythrocyte. Malaria is a significant problem for soldiers stationed in many areas of the world.
24.(U) Laboratory work and computer model building will proceed simultaneously. Laboratory studies will include measurement of (1) intermediates and enzyme level of glycolysis, the pentose cycle, the tricarboxylic acid cycle, and fatty acid synthesis; and (2) intermediates and enzyme levels of the purine salvage pathways. Computer models will be developed using a simulation language which permits the investigator to describe his model as a series of biochemical reactions with specified rate constants and initial substrate concentrations. The simulation program then sets up and solves a series of simultaneous differential equations describing the rates of change of concentration of each substrate in terms of rate constants and of concentration of other substrates.
25.(U) 77 10 - 78 09. Laboratory procedures have been established for measurement of intermediates of purine metabolism and glycolysis, for assay of each of the enzymes involved in these two areas of metabolism, and for identification of host and parasite isotypes of specific enzymes. A collaborative arrangement has been initiated with Dr. David Garfindel, an internationally recognized expert in simulation of intermediary metabolism. A data base has been accumulated for each of these two metabolic areas. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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PROJECT 3A161101A91C RESEARCH ON MILITARY DISEASES

Work Unit 106 Computer Simulation of Red Blood Cell Metabolism

Investigators

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Description:

Work was begun to develop a computer simulation model of intermediary metabolism in the erythrocyte, based on data both in existence and to be obtained in our laboratories. Work on this project to date has consisted of three major efforts: (1) establishing a data base, (2) obtaining enzyme and substrate data in our laboratory, and (3) establishing collaborative arrangements. Progress in each of these three areas will be discussed under "Progress". The remainder of this section will describe the general design of the study, the methodology, and the basic strategy of the malaria studies.

1. General design of the study

The basic question we are addressing in this work unit is: "By constructing separate computer models of the metabolism of uninfected erythrocytes, malaria-infected erythrocytes, and free malaria parasites, can we gain a better understanding of how the parasite utilizes the metabolic machinery of the host erythrocyte?" Two important practical goals this may help us attain are: (a) newer methods of metabolic attack on the malarial parasite, and (b) improved media for culture of the

parasites in vitro.

The computer model will initially focus on glycolysis and the pentose cycle, since LTC Haut and Dr Garfinkel have had their greatest amount of experience simulating this area of metabolism. Kinetic data for each enzyme, and concentrations of metabolic intermediates both during steady state and after perturbation will be obtained, when possible, from material published in well-reviewed journals. When such data are unavailble, they will be obtained in our laboratory or by personal communication from one of several acknowledged authorities in the field of erythrocyte metabolism. The model will essentially be set up to be consistent with what is known about kinetics of the individual enzymes and about steady state levels of metabolic intermediates. It will then be refined by making it agree with progressively larger numbers of experiments in which the cells have been perturbed and levels of metabolic intermediates have been measured.

When a reasonably accurate unlabeled model has been developed, a series of labeled models will be constructed. Each model will represent all the possible ways in which the isotopic carbon of a specifically labeled glucose (i.e., 1-^{14}C-glucose, 6-^{14}C-glucose) could be distributed in various metabolic intermediates as the label becomes randomized by shifts of one or more carbons in certain reactions (such as the transaldolase and transketolase reactions of the pentose cycle). Each reaction involving labeled compounds will have the same rate constant as the unlabeled counterpart. A single set of kinetic parameters will then be developed which will enable all of the labeled models to fit the literature data. The data base for the isotopic studies will include (1) standard studies, in which erythrocytes are incubated with labeled glucose, the $^{14}CO_2$ formed is trapped by hyamine hydroxide, and a time course is obtained by killing the reaction by addition of sulfuric acid to matched flasks at different intervals and counting the $^{14}CO_2$; and (2) studies in which $^{14}CO_2$ production is measured continuously in an ionization chamber with a vibrating reed electrode.

The second major emphasis of our simulation studies will be on the purine salvage pathways of the erythrocyte. Purine metabolism, particularly adenine nucleotide

metabolism, plays a significant role in maintenance of the malaria parasite in the host's red cell. The general approach to simulation of this area will be similar to that for glycolysis, but will lean more heavily toward simulation of the unlabeled system because of (1) the ease with which we can obtain nucleotide profiles by high pressure liquid chromatography, and (2) the fact that isotopic studies are not as helpful in this system as in glucose metabolism, where randomization makes it more difficult to obtain simultaneous fits of several labeled models unless the model is a good representation of the actual state.

2. Methodology

a. Computer simulation

The simulation language of Garfinkel (1,2) will be used to formulate the models. This simulation language permits the investigator to describe his model as a series of biochemical reactions. Input for the model consists of: (1) a series of reactions which define the system being simulated (e.g., glycolysis in the Ehrlich ascites tumor cell; mitochondrial metabolism in the hypoxic heart); (2) rate constants for both the forward and (where applicable) for the reverse reaction; and (3) initial concentrations for each chemical compound in the system. The simulation program first sets up a series of simultaneous differential equations describing the rate of change of concentration of each substrate in terms of rate constants and of concentrations of other substrates. It then solves the series of simultaneous differential equations and plots the concentration of each substrate as a function of time. At selected times, the computer will also print out first and second derivatives of each concentration, to indicate the rate of change of that particular concentration.

The data base for initial construction of the computer model will be (1) literature values for kinetic constants of each enzyme involved in red cell intermediary metabolism, and (2) literature values for substrate concentrations in the erythrocyte, both in the steady state and after various types of perturbation. From this data, and from previous simulation studies by Garfinkel, et al., both of individual enzymes (3-5) and

of the entire glycolytic pathway in specific tissues, such as Ehrlich ascites tumor cells (1,6), the perfused rat heart (7,8), the lactating rat mammary gland slice (9), and the perfused rat liver (10-12), a model in which each enzymatic reaction is described in detail will be constructed.

The model will then be refined by altering it so that it conforms to isotopic data as well. This will be done using the approach of Garfinkel in simulating use of radioactive tracers in brain metabolism (13,14). Matching models approximately describing the behavior of the biochemical system containing unlabeled compounds and the system with appropriate labeled inputs are constructed, and their behavior is compared with available experimental data. As the "unlabeled" model is usually much smaller than the "labeled" models, most of the manipulation is carried out with it, and the resulting kinetic parameters are repeatedly modified until the entire set of models closely approximates as much of the available experimental data as possible. Equations for the "labeled" models will be written by using a program that determines the labeling pattern of all metabolites after addition of a specifically labeled substrate to a specific unlabeled system (15).

Validity testing of the completed model will include examination of its ability (1) to conform to or explain data in the literature which were not used in construction of the model, and (2) to predict the results of experiments done in our laboratory or in other laboratories, in which the steady state metabolism of the erythrocyte is perturbed in a particular manner and metabolic changes are followed both with tracer studies and by measuring changes in levels of several key substrates.

b. Biochemical measurements

Glycolytic enzyme activities will be determined spectrophotometrically as described by Beutler (16). Glycolytic intermediates will be determined fluorometrically as described by Minakami(17-19). $^{14}CO_2$ production will be determined by a modification of the method described by Yunis (20).

Erythrocyte purine and pyrimidine nucleotide,

nucleoside, and nucleobase concentrations will be determined using high performance liquid chromatrography. Activities of enzymes of purine salvage and interconversion will be measured as described by Snyder et. al (21); overall de novo purine synthesis will be measured as described by Hershfield and Seegmiller (22).

c. Intermediary metabolism of malaria-infected erythrocytes

Malaria studies using the computer simulation model will focus on two experimental systems - P. knowlesi infection in rhesus monkeys, and P. falciparum grown in vitro in human erythrocytes.

Our department is currently involved in a study of metabolic alterations in erythrocytes of rhesus monkeys during the course of a synchronous P. knowlesi infection. In this study (WRAIR protocol # 012-74), purine nucleotides, glycolytic intermediates, purine salvage pathway enzymes, and glycolytic enzymes are all measured in samples taken at 4 hour intervals during two or three 24 hour cycles of patent parasitemia.

Models of glucose and purine metabolism of the uninfected rhesus monkey erythrocyte, the rhesus monkey erythrocyte infected with P. knowlesi, and the isolated P. knowlesi parasite will be developed. Construction of these three models should itself provide a significant amount of information about the relationship between the malaria parsite and the host cell. Once reasonable models of the steady state metabolism of the parasite, the cell, and the parasitized cell at one point in time have been developed, we will attempt to simulate the metabolism of a parasite developing intracellularly. Similar models will be developed for P. falciparum grown in vitro in human erythrocytes.

Progress

Work on this project to date has consisted of three major efforts: (1) establishing a data base, (2) obtaining enzyme and substrate data in our laboratory, and (3) establishing collaborative arrangements.

1. Establishing a data base

A major effort was made during the initial phase of this work to establish a data base on glucose and nucleotide metabolism by erythrocytes. Data from the literature was obtained by library research, and unpublished data has been solicited from a number of selected investigators in these two areas. Specific types of baseline information obtained include the following:

a. Substrate levels in human erythrocytes under various conditions

Steady state data for red blood cell glycolysis have been obtained from the literature. Of these, the most useful for our simulation studies are those by Minakami and Yoshikawa (19) and by Hamasaki et al (23). These data are particularly useful because they are detailed, examine the effects of specific incubation conditions on substrate levels (19), and show the time course of changes in substrate concentrations under specific conditions (23).

b. Kinetic data on specific enzymes

Accumulation of kinetic data on specific erythrocyte enzymes was facilitated by the extensive compilation of erythrocytic enzyme data by Friedemann and Rapoport (24). In view of the extensiveness of the enzyme data in that review, our efforts focused mainly on (1) obtaining the specific publications referred to in that review, and (2) examining the literature from 1974-1978. Specific types of information obtained included data on Vmax and Km, on various activators and inhibitors, and on the variation of activity with pH and temperature.

c. Isotope studies

Literature values were obtained for $^{14}CO_2$ production from 1-^{14}C-glucose, 2-^{14}C-glucose, etc). Two types of data were obtained.

The first type of data, the classic type, are obtained when cells are incubated with labeled glucose in special flasks, and the $^{14}CO_2$ liberated is trapped in a special chamber inside the flask filled with a substance such as hyamine hydroxide. These cells are usually incubated for one or more hours, then acid is

added to the red cells to stop their metabolism, and the hyamine with its bound $^{14}CO_2$ is removed and counted in a liquid scintillation counter. Extensive data obtained by this technique is available in the literature. A particularly detailed group of studies which will be used as a basic framework for this data is the work described by Yunis and Yasmineh (20).

A second type of data, in which $^{14}CO_2$ production from labeled substrate is measured continuously in an ionization chamber with a vibrating reed electrode, was described by Davidson and Tanaka (25). A moderate amount of literature data are available using this technique, but it is likely that we will have to obtain a significant amount of this data either by ourselves or through Dr Tanaka at UCLA (Harbor General Hospital), Dr Metz (Ohio State University), or Dr John Schwartz (formerly at WRAIR, now at the Thorndike Laboratories in Boston).

d. Specific information about localization of specific enzymes, and about physical proximity of certain enyzmes to specific other ones

This literature search was aided significantly by the recent review by Schrier (27) on human erythrocyte membrane enzymes. A particular area of interest in this regard is the observation by Fossel and Solomon (27) that glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase (PGK), and diphosphoglycerate mutase (DPG-M) appear to bind tightly together on the membrane, so that the flow of 1,3-diphosphoglycerate through the competing reactions PGK (which produces ATP to drive the cation pumps) and DPG-M (which produces 2,3-DPG) is closely controlled.

e. Data on erythrocytes with specific enzyme deficiencies

Data on erythrocytes with specific enzyme deficiencies have been obtained by review of published reports on patients with specific disorders. These data include (1) substrate levels in these erythrocytes following incubation under standard conditions, (2) kinetic data on the abnormal enzymes, with particular emphasis on their control by specific activators and

inhibitors, and (3) isotope studies. The data on enzyme-deficient erythrocytes are important for analyzing the role of particular enzymes in overall control of erythrocyte metabolism.

2. Obtaining enzyme and substrate data in our laboratory

Work in this area falls into four categories: (a) establishment of methods: (b) studies in normal and enzyme-deficient erythrocytes; (c) studies in P. knowlesi infected rhesus monkey erythrocytes; and (d) studies in P. falciparum-infected human erythrocytes.

a. Establishment of methods

Methods have been set up in our laboratory for the following: (1) measuring the activity, under optimal conditions, of the majority of the glycolytic enzymes and of the enzymes of purine salvage and interconversion; (2) obtaining profiles of the purine and pyrimidine nucleotides, nucleosides, and nucleobases in whole blood extracts (which primarily reflect erythrocyte values) using high performance liquid chromatography; (3) measuring the level of the major glycolytic intermediates, using enzyme-based fluorometric assays; (4) measuring $^{14}CO_2$ production by erythrocytes, using both incubation (20) and vibrating reed (26) techniques; and (5) starch gel electrophoresis of several rate controlling or otherwise important glycolytic enzymes (G6PD, hexokinase, phosphofructokinase, and pyruvate kinase) and of several enzymes involved in purine metabolism.

Work is currently in progress to set up additional methods, including: (1) obtaining profiles of glycolytic intermediates using high performance liquid chromatography; and (2) measuring the activity of enzymes of pyrimidine metabolism and of de novo purine synthesis.

b. Studies in normal and enzyme-deficient erythrocytes

Enzyme, substrate, and ^{14}C-substrate incubation studies of glycolytic metabolism have been standardized for normal human erythrocytes in our laboratory. In addition, several patients with deficiency of G6PD or pyruvate kinase have been studied in a similar manner.

$^{14}CO_2$ production from 1-^{14}C-glucose and 6-^{14}C-glucose was studied, using the vibrating reed electrode system, in red cells from normal subjects and from patients with G6PD deficiency before and after stimulation with methylene blue.

c. Studies in P. knowlesi-infected rhesus monkey erythrocytes

Enzyme levels of a number of purine salvage and interconversion enzymes (APRT, HGPRT, ADA, PNP, AK, and PRPP-S) and of glycolysis (G6PD, HK, PFK, PK) have been measured in uninfected and in P. knowlesi-infected rhesus monkey erythrocytes. Levels of the glycolytic enzymes were measured at 4-6 hour intervals during the course of patent infection in six monkeys, to determine whether the levels of any of the enzymes varied with the stage of parasite intraerythrocytic development. Studies on both groups of enzymes showed selective increase of some enyzmes, but not of others, suggesting that certain enzymes were present in the parasite, whereas others were not. The sequential studies of the glycolytic enzymes indicated that enzyme levels did not vary with stage of parasite maturation. For those enzymes whose level increases significantly during infection, we have set up starch-gel electrophoresis separations to differentiate host enzyme from parasite enzyme. We plan to isolate the parasite enzymes so that their kinetics can be studied in the same detail as the host enzymes.

d. Studies in P. falciparum-infected human erythrocytes

Because of the extensive data available on human erythrocytes, and the clinical importance of P. falciparum as a human pathogen, we are examining the metabolic aspects of P. falciparum cultured in vitro in human erythrocytes. So far, our examination of this system has been focused on examination of levels of purine metabolizing enzymes and isolation of parasite-specific enzymes. However, we plan to examine this system in as much or greater detail than the rhesus erythrocyte P. knowlesi system.

3. Establishing collaborative arrangements

Collaborative arrangements for development of the proposed model of erythrocyte metabolism have been established with Dr David Garfinkel, Professor of Computer Information Science at the Moore School of Electrical Engineering, University of Pennsylvania and with Dr Zelda B. Rose, Associate Member, Institute for Cancer Research, Fox Chase. Dr Garfinkel has played a key role in the development of digital computer simulation as a method of examining biochemical systems. He is probably the foremost authority on computer modeling of biochemical systems in America. Dr Rose is an internationally known investigator in the field of erythrocyte metabolism, who has made a number of fundamental observations in this research area.

An indication of Dr Garfinkel's interest in this project is the inclusion in one of his recent NIH grant renewal requests of plans for utilization of artificial intelligence techniques to construct a model of erythrocyte glucose metabolism in collaboration with our laboratory and Dr Rose's.

Project 3A161101A91C RESEARCH ON MILITARY DISEASES

Work Unit 106 Computer Simulation of Red Blood Cell Metabolism

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OC 6440	78 09 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	K. Completion	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	206
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*

(U) Factors Influencing Secretory States

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003800 Life Support 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:* NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 77	2	75
FISCAL YEAR CURRENT 78	1	50

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Hyperacidity; (U) Gastric Mucosa Barrier; (U) Stress Ulcers; (U) Histamine; (U) Bile Acids, (U) Duodenal Acid Handling

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23 (U) The technical objectives have been to investigate the mechanisms by which bile and acid injure the stomach and to investigate the mechanisms by which the duodenum removes acid from its lumen. Bleeding from gastric stress ulcers is an important problem for military combat surgery because up to 10 percent of combat casualties develop clinically detectable bleeding from gastric erosions.

24 (U) Gastric injury was measured using canine fundal pouches. Solutions containing acid and bile were circulated through the pouches and mucosal electrical potential difference and permeability were measured using a voltmeter and pH stat respectively. Duodenal acid removal was measured using a pH stat while carbon dioxide was measured using a mass spectrometer.

25 (U) 77 10 - 78 09 Different bile acids were found to have different effects on the gastric mucosa, rather than having the same effect as most clinicians have believed. Part of this could be explained by physical chemical properties of the bile salts. This may explain why bile reflux seems to cause ulceration in some patients but not in others. Acid loss in the canine duodenum was found to result from bicarbonate neutralization of acid. This mechanism could account for about 50 percent of the acid disappearance observed. Gastric back diffusion of acid did not involve bicarbonate. The ability of the duodenal mucosa to remove acid has been unrecognized until recently and may be an important defense mechanism to protect against gastric ulceration.

For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 206 Factors Influencing Secretory States

Investigators
Principal: John W. Harmon, MC

I. Gastric Bile Injury - Gastric Stress Ulcers.

A. Background and statement of problem. Bile, with ischemia and acid, have been implicated in the etiology of gastric stress ulcers. Gastric stress ulcer bleeding is a major cause of morbidity and mortality among combat casualties. Our laboratory has studied the effects of various bile acids on the gastric mucosa of dogs to more precisely understand their injurious effects.

B. Experimental approach. Dogs were prepared with chronic Heidenhain pouches with 2 cannulas each for through and through perfusion. Acid loss was measured with a pH stat and transmucosal electrical potential difference was measured with a voltmeter, KCL agar bridges, and Calomel electrodes.

Reduction in potential difference and increased permeability to hydrogen ion were the indices of injury measured.

C. Results. Different bile acids were found to have different capacities to injure the gastric mucosa. Further these effects were pH dependent. The table below shows the net acid loss in uEq/10min and potential differences in MV from a series of these experiments. Increasesin acid loss (e.g. back diffusion) and decreases in potential difference are signs of damage to the gastric mucosa.

Bile Salt Conc (mM)	Cholate	Taurocholate	Deoxycholate	Taurodeoxycholate
	(Acid loss from pouch lumen - uEq/10min)			
0	35±8	22±9	10±10	16±6
5	52±13	18±8	66±20	237±50*
10	32±12	247±22	54±25	325±75*
20	47±11	249±18*	29±14	445±106*
	(Potential difference mV with lumen negative)			
0	47±2	51±2	54±1	50±1
5	40±4	52±1	50±1	14±1*
10	39±4	14±1*	50±2	14±1*
20	36±5	13±2*	51±1	13±1

(*$p < .01$ different from control)

In a second series of experiments the effect of these four bile salts on gastric potential differences at pH7 was evaluated.

	Cholate	Taurocholate	Deoxycholate	Taurodeoxycholate
		(Potential difference mV)		
Control pH7 ATS	54±1	56±2	54±2	54±2
5mM Bile Salt	19±2*	58±4	20±5*	11±3*

D. Conclusions and recommendations. The toxicity of the different bile salts is different at pH 7 than at pH 2. It is therefore apparent that the exact bile salt composition as well as the pH of reflux fluid may determine whether bile reflux will damage the gastric mucosa.

II. Bile injury to the esophagus.

A. Background. Esophageal reflux with resulting esophagitis and stricture is a major gastrointestinal disease. The role of bile in damaging the esophageal mucosa is not well understood. A series of experiments were undertaken to look for differences in the injurious effects of various bile acids. If differences were found this could explain why some patients with bile reflux developed esophagitis, while others did not.

B. Experimental approach. Rabbits anesthetized with Innovar were studied after cannulation of their proximal and distal esophagus using a recirculating system incorporating a pH stat and autoburette. The system was calibrated daily. Net acid loss from the lumen of the esophagus was measured at 10 minute intervals for one hour long periods. Four rabbits were tested in each of five groups. Perfusates of pH 2 made isotonic with NaCl were tested alone and then with 5mM bile salt. With bile present esophageal pH was then raised to pH 7 for ore hour after which another period of pH2 perfusion was carried out. Because no acid back diffusion occurs at pH 7 without a pH gradient, the effect of pH 7 bile exposure was only evident in the subsequent pH 2 period.

C. Results. Differences in the injurious effects of various bile acids were clearly evident in the experiments as shown in the table below. (See Table II)

TABLE II

Net acid loss in uEq/10min is shown.

Bile acid (pKa)	Control at pH 2	At pH 2 with bile	At pH 2 after bile at pH 7
None	2±1	1±1	3±1
Cholate (5.0)	3±1	5±2	13±2*
Taurocholate (1.9)	9±5	72±12*	76±10**
Deoxycholate (5.3)	2±1	3±2	55±11**
Taurodeoxycholate (1.9)	1±1	51±7**	47±6**

(*$p < .05$), **$p < .01$ bile of control by Student's paired t test

D. Conclusions and recommendations. It is evident from these data that these bile acids have differing effects on the hydrogen ion permeability of the rabbit esophagus related at least in part to their pKa. The bile acids with pKa less than 2 were damaging to the mucosa at pH2, while those with pKa above 2 were not. All of these bile salts increased esophageal permeability to hydrogen ion after bile exposure at pH7.

III. Recovery of the Gastric Mucosa from Bile Induced Injury.

A. Background. The permanence of bile induced injury to the gastric mucosa has not been well studied. Bile reflux tends to be episodic clinically and this allows intervals for recovery of the mucosa between the periods of exposure of bile. The time course of recovery was studied at 3 different levels of acidity to determine the importance of acidity in this phenomenon.

B. Experimental approach. The recovery from a bile salt induced injury was studied in 3 mongrel dogs with Heidenhain pouches. Net acid back diffusion (NABD) was measured and transmucosal electrical potential difference (PD) measured by usual methods. The effects of exposure to 5mM taurodeoxycholate at pH 2 for 40 minutes were studied. This exposure increased net acid back diffusion from 23±5 to 184±16 uEq/10min ($p < .01$) and reduced PD from 56±2 to 18±2 mV ($p < .01$). After injury the pouches were copiously irrigated with saline to remove bile and then recovery was observed as the pouches were perfused with pH 1, 2 or 7 HCl solutions made isotonic with NaCl. The recovery values were compared with same dog's preinjury values at the same pH perfusion.

C. Results and discussion. Net acid back diffusion and transmucosal electrical potential differences from a series of experiments is shown below.

AFTER BILE SALT INJURY

	Control pre-bile	Bile at pH 2	0-30 min	30-60 min	60-90 min
pH 7	-69±35	185±35	-37±5	29±5	-34±13
pH 2	13±3	199±29	180±34**	46±7*	23±11
pH 1	9±3	167±28	450±119**	239±51	345±52
	(Transmucosal Electrical Potential Difference, mV)				
pH 7	62±5	21±4	22±4*	46±6*	61±2
pH 2	56±3	17±3	30±5*	51±3	59±2
pH 1	48±3	16±2	29±2*	37±2**	39±4*

Student's paired t test, *$p < .05$, **$p < .01$)

D. Conclusions and recommendations. These data show that at pH 2 and pH7, PD and NABD returned to preinjury levels within 90 minutes after bile injury. At pH 1 however recovery did not occur. The pH dependence of recovery from bile induced injury may have relevance to patients with episodic reflux of bile into their stomachs.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 206 Factors influencing secretory states

Literature cited.

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Project 3M161102BS01

RESEARCH ON MILITARY INJURY AND DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6431	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	121
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)
(U) Ecology and Control of Disease Vectors and Reservoirs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002600 Biology 005900 Environmental Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA
EXPIRATION:
b. NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	6	276
FISCAL YEAR CURRENT 79	6	318

19. RESPONSIBLE DOD ORGANIZATION
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NAME: Roberts, MAJ L. W.

21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Arboviruses; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Control; (U) Trombiculid mites

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Studies emphasize control of vectors of arbovirus, rickettsial and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures.

24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Seasonal changes in size of and infection rates in vector populations are determined; biological processes of vector species, such as pathogen transmission, diapause and reproductive physiology, are studied in the laboratory.

25. (U) 77 10 - 78 09 Four strains of arboviruses, tentatively identified as St. Louis encephalitis virus, were isolated from overwintering Culex pipiens collected from Delaware, Maryland and New Jersey during February and March 1978. Field studies on winter survival of C. pipiens indicated that a significant proportion (12.3 per cent) given a prehibernation blood meal before release into hibernacula survived the winter. One of eleven sentinal goats placed in the Pocomoke Cypress Swamp developed antibodies to Jamestown Canyon virus. The vector responsible for transmission of this virus has not yet been identified. Experiments with strains of Rickettsia tsutsugamushi in colonies of Leptotrombidium arenicola and L. fletcheri indicate that the two strains differ widely in virulence for laboratory animals. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 121 Ecology and Control of Disease Vectors and Reservoirs

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Description

This task involves field and laboratory studies of the relationships between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanisms and the development of improved methods of control of arthropods of medical importance.

Progress

1. Overwintering Ecology of St. Louis Encephalitis (SLE) Virus

Previous studies of overwintering Culex pipiens in Maryland, Pennsylvania and New Jersey resulted in the isolation of two strains of SLE virus from hibernating mosquitoes of that species (1). The question remained as to how these mosquitoes became infected. Previous studies (2) have provided a biologically plausible model for the natural occurrence of blood feeding in prehibernating Cx. pipiens. During the winter of 1977-78 an investigation was undertaken to determine whether bloodfed diapausing Cx. pipiens could survive a winter when released into natural hibernacula.

Three groups of mosquitoes with different physiological histories were used in this experiment:

1) "Wild" mosquitoes which had entered abandoned ammunition bunkers within a one week period prior to the release date.

2) "Unfed, lab reared, diapausing" mosquitoes

3) "Bloodfed, lab reared, diapausing" mosquitoes

The mosquitoes in groups 2 and 3 were from a colony established in 1975 from hibernating females collected from Fort Mott, New Jersey. Larvae for these two experimental groups were reared in our insectary using conventional techniques at a temperature of 27°C and a daily photoperiod of L:D, 9:15 (hr light: hr dark per 24-hr period). At the beginning of pupation the temperature was lowered to 15°C. Pupae were randomly selected and placed in two different bioclimatic chambers programed for 15°C, L:D 9:15 and 80-90% relative humidity. Seven days after adult emergence the temperature in the bioclimatic chamber where group 3 mosquitoes was being held was raised to 27°C, and these mosquitoes were given a bloodmeal on 1-2 day old chicks. All fully engorged females from group 3 were removed and placed back into the 15°C, L:D 9:15, 80-90% relative humidity environment, where they were held for from 6-10 days to allow for digestion of the bloodmeal.

On 14 November 1977 a total of 547 Cx. pipiens (209 "wild", 200 "unfed lab-reared" and 138 "bloodfed lab-reared") were released inside each of two abandoned ammunition bunkers at Fort Washington, Maryland. Each group of mosquitoes was marked with a disginguishing fluorescent dust. Counts of the released mosquitoes were made twice weekly with the aid of a portable "black light".

Within the first week after release the numbers of marked mosquitoes had decreased by 40 to 50% in Decator bunker (Fig. 1) and by 80 to 90% in Emory bunker (Fig. 2). Since no dead marked mosquitoes were found in either bunker, it is believed that this initial drop in numbers was due to emigration from the release sites. By the end of November the number of "wild" and "unfed lab-reared" mosquitoes in Decator bunker had stabilized at about 40 percent of the original number released. However, the "bloodfed lab-reared" mosquitoes continued to decline in numbers until mid January 1978 and then stabilized at approximately 20% of the number originally released, until 3 March when the study was terminated. The similarity in rates of recovery of the "wild" and "unfed lab-reared" mosquitoes suggests that there was no significant difference between the physiological condition of the two groups at the time they were marked and released. The lower percentage of survival in the bloodfed group was expected; only those mosquitoes which displayed ovarian diapause after the bloodmeal survived, while mosquitoes which developed eggs following the bloodmeal probably died because of the loss of hypertrophic fat.

There was a distinct difference in environmental conditions as measured by temperature and relative humidity between the two bunkers (Figs. 1 and 2). The overall fluctuations in both temperature and humidity were generally lower in Emory bunker where total mosquito survival was less than 10 percent. The average weekly

high temperature in Decator bunker was below freezing from 1 February through 3 March. At no time during the study was the average weekly high temperature in Decator bunker below freezing. At the termination of the study on 3 March, 34 of the 276 bloodfed females released the previous November were found. Of these, only 1 had fully developed eggs in its ovaries, while the rest were nulliparous. These data support the hypothesis that a significant number of mosquitoes which have taken a prehibernation bloodmeal can survive the winter.

2. St. Louis Encephalitis Virus (SLE) Isolation from Overwintering Culex pipiens

A total of 1720 hibernating Cx. pipiens was collected during March 1978 from 4 different abandoned U.S. Army forts located in Maryland, New Jersey and Delaware (Table 1). In order to terminate diapause, all mosquitoes were held in a bioclimatic chamber at 25°C, L:D, 15:9 and 80-90% relative humidity for from 14 to 25 days. After the holding periods, the mosquitoes were given a bloodmeal on 1-2 day old chicks. Prior to these feeds each chick was bled and its blood, diluted 1:10 in Medium 199 plus Hanks' base, inoculated IC into a litter of 3-5 day old mice to eliminate the possibility that the chicks served as virus sources for the mosquitoes. Likewise, each chick was held for 14 days after the mosquitoes had fed and then tested for the presence of SLE antibody. All mosquitoes that engorged on the chicks were segregated and held an additional 10 days until blood digestion was completed and then assayed fir virus. Four strains of virus lethal for suckling mice were isolated from hibernating Cx. pipiens, 2 from mosquitoes collected at Ft. Washington and 2 from Ft. Mott mosquitoes. Three of these isolates came from mosquitoes given a posthibernation bloodmeal, while the fourth was from mosquitoes held for 25 days at 25°C following collection. Although the identification of these viruses is not complete, it is likely that all four are SLE virus on the basis of mouse mortality, morphology of plaques in cell culture and the fact that they came from Cx. pipiens. The rate of isolation of these strains was 1 per 430 mosquitoes tested. None of the chicks fed upon by mosquitoes from the positive pools developed antibodies to SLE.

3. Ecology of Keystone (KEY) and Jamestown Canyon, (JC) Viruses

Two viruses of the California encephalitis group, Keystone (KEY) and Jamestown Canyon (JC), are enzootic on the Del Mar Va peninsula. Aedes atlanticus has been clearly shown to be the principal vector of KEY virus in the Pocomoke Cypress Swamp (PCS)

in a cycle that involves gray squirrels and/or cottontail rabbits (3). This mosquito has also been demonstrated able to transmit KEY virus transovarially to its offspring (4). In 1975 cottontail rabbits trapped on Assateague Island, Virginia were found naturally infected with KEY virus, although neither Ae. atlanticus nor gray squirrels are known to occur on the island (3). Two of the most abundant mosquito species on Assateague, Ae. sollicitans and Ae. tarniorhynchus, have been experimentally infected with KEY virus, but it is not known whether either can transmit the virus to susceptible hosts (3). During the summers of 1976 and 1977 extreme conditions of drought occurred in the PCS and prevented the emergence of Ae. atlanticus populations. Nevertheless, antibodies to KEY virus were found in the sera of gray squirrels trapped in the PCS in 1976 and 1977, and it is assumed that at least some of the KEY immune-animals were infected during those years. It thus seems likely that either a mosquito other than Ae. atlanticus (possibly salt marsh mosquitoes such as Ae. sollicitans or Ae. taeniorhynchus or some other blood-feeding arthropod (e.g., tabanids and/or ticks) is involved in the cycle of KEY virus.

Infections with JC virus are widespread in White-tail deer on the Del Mar Va Peninsula, for between 1972 and 1974 antibodies to this virus were detected in sera from 97/182 deer (53%) in that area (5). Association of JC virus with a particular vector species, however, is not so clear, for only 2 isolations of this virus (one each in 1971 and 1972) have been made from mosquitoes on the Del Mar Va peninsula (5). Although both isolations came from Ae. canadensis collected in the PCS, the very low infection rates (1:42,434 in 1971 and 1:63, 216 in 1972) observed in that mosquito suggest the possible involvement of other vector species in the JC cycle in the PCS.

Our primary objective in the PCS was to identify the vector of JC virus. In an effort to accomplish this, 5 magoon traps were operated in different habitats--including swamp, upland forest, and open fields (Fig. 3). Two JC virus antibody-free goats were placed in each trap. All blood-feeding insects collected in the traps were stored at -70°C for future identification. Goats were bled weekly from May through mid-September. If JC antibody is detected in any of the goats, blood sucking arthropods collected the previous month from its trap and adjacent light trap will be tested for virus. To date 180 collections have been made from the magoon traps. During a period of high mosquito density (6-27 June) both morning and evening collections were made once a week from each Magoon trap to determine if the various

species entered the trap during daylight or darkness. Identification of the mosquitoes and tabanids collected in the traps has not been completed. To date, sera collected from goats through 20 July have been tested. Neutralizing antibody to JC virus was detected, by plaque reduction neutralization test in BHK 21 cell cultures, in sera collected from a goat, housed at Dorsey's Pasture (Fig. 3), on 6 and 13 July. The percent reduction of plaques by a 1:10 dilution of the sera was 83% for JC and 20% for KEY on 6 July, and 100% for JC and 90% for KEY on 13 July.

Surveillance for KEY-and JC-virus-infected mosquitoes was also maintained by operation of 10 CO_2-baited, battery-powered light traps in the PCS study area in all habitats (Fig. 3). A total of 181 trap-night collections were made from the 10 sites in the PCS during 1978. Mosquitoes and tabanids from these collections were stored at -70°C for future identification and virus assay.

Unusually dry weather prevailed on the Delmarva peninsula in 1976 and 1977 (3). Adults of the principal Keystone virus vector, Ae. atlanticus, were present in low numbers in the PCS and Snow Hill areas in 1976 and absent in 1977. After two years of weather conditions extremely unfavorable to mosquitoes, it was of interest to determine if prevalence of antibody to JC and KEY virus had declined in feral vertebrate populations in those areas.

Sera from white-tailed deer were collected from carcasses brought in for registration with Maryland State Game officials during the hunting season in November 1977 at Snow Hill, MD. Squirrels were trapped and bled twice weekly in the PCS in 1977 and 1978. In 1977, 76 percent of sera from deer (152/200) and 45 percent of sera from gray squirrels (29/64) tested had neutralizing antibody for JC and/or KEY viruses.

In 1975 and 1976 antibodies to KEY virus were detected in 30 and 25 percent, respectively, of all sera from squirrels trapped in the PCS (3). In 1978, 44 sera were collected from 33 squirrels trapped in the PCS. Antibodies to KEY virus were detected in sera collected from one squirrel on 5, 12 and 18 July. Percentage reduction of KEY and JC virus plaques were 99 and 18 percent, respectively, for the 5 July serum, and there was 100 percent reduction of KEY virus plaques and 88 percent reduction of JC-virus plaques by the 13 July serum.

4. Larval population dynamics of Ae. atlanticus

Larval population dynamics of Ae. atlanticus were studied in the PCS to estimate frequency of hatches, larval abundance and survivorship in the field.

In order to estimate abundance of Ae. atlanticus larvae, an area sampler described previously (2) was refined and calibrated. The efficiency of the sampler was assessed in the laboratory employing 2nd and 4th instar Ae. infirmatus larvae under the various temperatures, water depths and larval densities observed in the field. Sampler performance was not affected by temperature or larval density; fourth instars were captured more frequently than expected, and efficiency for both larval age classes increased from 50% at 2 cm water depth to 100% at 15 cm. Estimates of larval abundance can be corrected for sampler bias.

The Ae. atlanticus breeding area in the PCS is normally flooded throughout the winter. Water recedes in the spring, and any subsequent flooding may result in a hatch of Ae. atlanticus. Consequently, during 1978 water samples were routinely dipped from newly formed ground pools and examined for Ae. atlanticus larvae. When larvae were found the number of larvae per m^2 in selected ground pools was estimated daily using the area sampler. When Ae. atlanticus larvae were present water temperature was monitored constantly, and pool size and average depth were measured when larval samples were taken. When pupation commenced, a large emergence trap (3 X 5 X 2m) was suspended over the ground pool and checked daily for emergent adults.

Three hatches of Ae. atlanticus occurred in 1978. The first followed 3.3 cm of rain on 9 May. The deepest ground depressions in the breeding area still contained water from the winter inundation; however, slightly higher, peripheral areas had dried and were reflooded by this rainfall. Second instar Ae. atlanticus were observed on 12 May in small, shallow isolated ground pools at the Atlanticus North site (Fig. 3). By 18 May the majority of larvae were fourth instars and densities in the ground pools ranged from zero to 124 Ae. atlanticus larvae per m^2. Sampling was halted on 19 May since abundance was too low to obtain reliable estimates of population sizes or survivorship.

A major hatch occurred after 6.2 cm of rain fell on 10 June. Estimations of larval abundance were limited to a 4000 m^2 section of the Atlanticus North study area (Fig. 3). Within this section a 100 m^2 study area was chosen for daily sampling. On 13 June 55% of the Atlanticus North area was flooded. Water gradually receded to a 10% inundation by 19 June. At the 100 m^2 site, mean water

depth and pool size were 12 cm and 25 m^2, respectively, on 13 June. The pool was completely dry by 21 June. Mean daily water temperature ranged from 15 to 21°C (Fig. 4).

The following discussion applies only to the 100 m^2 site. When sampling was begun on 13 June, most Ae. atlanticus larvae were third instar, and the mean total larval density was 225/m^2 (Fig. 5) or a total population in the pool of 5700 larvae. The pool decreased gradually and maximum numbers of 4th instars were observed on 17 June. Total larval density was 650/m^2, and total number of larvae in the pool was 7000. This increase in total number of larvae is within sampling error, however. After this date the population declined precipitously. Although a few pupae were observed on 18 and 19 June, no immature Ae. atlanticus were found on 20 June. The large emergence trap collected 3 Ae. atlanticus males on 21 June and one male and female each on 22 June. No other adults emerged from this pool. Note that while larval numbers/m^2 increased from 13 to 17 June, the total number of larvae in the pool was relatively constant.

Finally, between 24 July and 1 August, 14 cm of rain fell in the PCS. Flooding was extensive in the breeding area. While no Ae. atlanticus larvae were observed, a few adults were seen in Magoon trap collections made on 15 August.

5. Laboratory colonization Ae. atlanticus

During the week of 12-16 June approximately 5,000 Ae. atlanticus larvae were collected from ground pools around the "Atlanticus North" site in the PCS. The larvae were transported to WRAIR for colonization attempts.

The overall objective to establish a laboratory colony of Ae. atlanticus was not achieved, however, various stages in accomplishing this goal were successful. The larval and pupal rearing techniques resulted in minimal mortality. The major mortality was observed in adults 3 days after a variety of emergence cages had been set-up in the insectary. Both males and females appeared to suffer equal mortality, however, since the males emerged before the females the majority of deaths were in males. These cages were maintained at a temperature of 31-32°C and a 70-80% RH. There appeared to be uniform mortality in the various types of cages. In contrast, however, the adults placed in a 22°C incubator, suffered no mortality. The mortality of adults in the insectary was attributed to dessication and therefore, to increase the humidity, each cage was covered with a wet towel and then a plastic bag was placed over the entire cage. This modification appeared to rectify the problem because massive adult mortality was not observed thereafter.

A shortage of males resulted from the early die off and created concern about the physiological state and mating competency of the survivors. Only 1 of 10 females dissected from various cages, contained sperm in their spermathecae. There did not appear to be any problem in getting the females to take bloodmeals and egg production began approximately 5 days after engorgement. The females did not appear to be very selective for oviposition substrates, because eggs were laid through the screen on the underside of damp towels placed on top of the cages as well as on the 3 types of substrate placed inside the cages. A total of 2,249 eggs was obtained. The majority of these eggs came from females in 1 ft^3 cages. All the eggs were conditioned for 7 days and then hatching was attempted by placing the eggs in a solution of yeast and distilled water. None of these eggs had hatched after 48 hrs. The eggs were then cleared in a 5% clorox solution, and it was determined that none were fertile. The majority of the remaining females were then dissected and only 1 female was found to be inseminated.

6. Keystone Virus Studies on the Chincoteague National Wildlife Refuge (CNWR)

To identify the vector of Keystone virus on the Chincoteague National Wildlife Refuge (CNWR), antibody-free sentinel hamsters were placed at 10 sites in the CNWR to detect KEY virus citculation (Fig. 6). At 5 of these sites 2 hamsters were kept within a hanging net mosquito trap; the other 5 sites each had 2 sentinel hamsters in close proximity to a CO_2-baited battery-powered light trap. The sentinel hamsters were replaced biweekly and held for an additional 2 weeks before being bled to determine presence or absence of antibody to KEY and JC viruses. Blood-feeding insects were removed from the light and net traps semiweekly and stored at -70°C.

A total of 77 collections from the 5 net traps and 164 trap-night collections from the 5 light traps were made from the 10 sites in the CNWR during 1978. Identification of mosquitoes and tabanids in these collections and sorting into pools for virus assay have not yet been completed. A total of 17 collections of mosquitoes were made from 2 artificial resting boxes in the CNWR. These collections yielded a larger number of anopheline mosquitoes, many of which were engorged, than did either the net-traps or light traps.

In order to confirm the presence of KEY virus antibody in the local population of cottontail rabbits a trap, mark, and release program was initiated in mid August 1978. To date serum samples have been collected from 5 different rabbits. The serum samples were stored at -20°C for future assay.

Ae. infirmatus has been naturally infected with Keystone and Trivittatus viruses in Florida (6), and is closely related to Ae. atlanticus, a known vector of KEY virus. In recent years, antibodies to KEY virus have been detected in wild-caught rabbits in the CNWR and in the PCS. The arthropod vector of this virus is unknown in the CNWR. Previous attempts to isolate viruses from dominant island species, Ae. taeniorhynchus, Ae. sollicitans, and Ae. canadensis, have yielded no KEY virus isolations. The recent discovery of a large breeding population of Ae. infirmatus in the CNWR has stimulated inquiry into the bionomics of this potential vector species.

The immatures of Ae. infirmatus were collected in large numbers in the fresh-water swamp in association with the following species of mosquitoes: Ae. canadensis, Ae. vexans, Cx. salinarius, Cx. territans, Phorophora ferox, and Ps. howardii. During a period of elevated larval densities (20 July) approximately 6,000 larvae were collected from a small portion of the swamp, of which 3,000 were Ae. infirmatus. The general habitat in this swamp is characterized as a secondary deciduous forest of primarily laurel, sweet gum, maples, and oaks. In and around the margins of the swamp, the understory is mostly greenbriar and ferns. The larval mosquito habitat is partially to completely shaded. The water is temporary, stagnant and light yellow with abundant organic material of mostly leaves and detritus, and with little or no algae. A total of 108 pools each containing 25 3rd and 4th instar Ae. infirmatus were assayed in 3-5 day old mice, but no agents were isolated.

7. Systematics of Del Mar Va Mosquitoes

The systematics and bionomics of most of the species and species groups of mosquitoes in North America are poorly known, and significant nomenclatorial and taxonomic changes in these taxa can be anticipated in the future. During 1978 attempts were made to individually rear the immatures of the mosquito species occurring in the PCS and on Assateague Island in order to provide a reference collection for present and future studies, and to have voucher specimens on hand for species confirmation in the event of systematic changes. Immature collections were made at or near the following sites in the PCS: Atlanticus North, 200, Slab Rd., Pasture Point, River, and Sawdust Pile (Fig. 3). These collections came from a variety of habitats such as ground pools, ponds, tree holes, artificial containers, swamps, marshy depressions, and stream margins. A total of 439 individual rearings (267 larval, 134 pupal, 38 incomplete) were made for 1145 specimens of the following species from the PCS: Aedes (Ochlerotatus) atlanticus, Ae. (Och.) cantator, Ae. (Och.) triseriatus-hendersoni, Anopheles (Anopheles) bradleyi, Culex (Culex) restuans, Cx. (Neoculex) territans,

Culiseta (Climacura) melanura, Cu. (Culiseta) inornata, Orthopodomyia signifera, Psorophora (Janthinosoma) ferox and Ps. (Psorophora) howardii. The dominant mosquitoes from the above list occurring in the PCS were Ae. canadensis and Ps. ferox, and to a lesser degree Ae. atlanticus and Cu. melanura.

Larval mosquitoes were also collected for rearing from brackish inlets and the fresh-water swamp on the Chincoteague National Wildlife Refuge (CNWR). A total of 260 individual rearings (182 larval, 64 pupal, 14 incomplete) were made for 702 specimens of the following species: Aedes (Aedes) cinereus, Ae. (Aedimorphus) vexans, Ae. (Ochlerotatus) canadensis, Ae. (Och.) infirmatus, Anopheles (Anopheles) bradleyi, Culex (Culex) restuans, Cx. (Cul.) salinarius, Cx. (Neoculex) territans, Psorophora (Janthinosoma) ferox, and Ps. (Psorophora) howardii. The dominant mosquitoes in the salt marsh ecosystem which were not collected were Ae. taeniorhynchus and Ae. sollicitans. In the fresh-water swamp the dominants collected were Ae. infirmatus, Ae. canadensis, and Ps. ferox, and somewhat Cx. territans and Ps. howardii.

8. Studies of Rickettsia tsutsugamashi in vector mites

It has long been known that Rickettsia tsutsugamushi is transmitted vertically (transovarially) by vector mites. Work done in Malaysia has shown that this transmission may be highly efficient. It has been demonstrated that although previously noninfected larval mites can take up R. tsutsugamushi during feeding on a rickettsemic host, subsequent generations of larvae do not appear to be infected indicating that the mite is both reservoir and vector of R. tsutsugamushi. To obtain a better understanding of the vector-host-pathogen interrelationships, we initiated laboratory studies of infected and noninfected vectors obtained from Malaysia.

An efficient mass rearing technique, based on that described by Traub et al. has been developed for both normal Leptotrombidium spp and those infected with Rickettsia tsutsugamushi.

Except during the larval feedings, mites in all stages of development are maintained in clear, screw cap glass jars, 5 cm diam X 6 cm high. Each jar has a 2.5 cm thick bottom layer of a hardened mixture of 9 parts plaster of Paris to 1 part activated charcoal (8). Rearing jars are autoclaved and reused after transferring chiggers to clean jars to minimize problems with mold growth. Nymphal and adult mites are fed twice per week with eggs of the collembolan Sinella curviseta Brook, with 5 or more eggs per mite provided at each feeding interval. The twice weekly feedings with S. curviseta eggs minimizes losses of mite eggs and quiescent stages due to predation (cannibalism) by nymphal and adult mites.

To provide eggs, the S. curviseta are reared in covered styrofoam cups having a 2.5 cm hardened layer of the plaster-charcoal mixture. Water is added to the substrate to maintain a high humidity, and powdered yeast provided as food for the collembola. Containers are changed at weekly intervals to enhance egg production. All stages of mites are maintained in a Freas Model 818 incubator, modified to include a low limit temperature control as a safeguard against heating unit failure, and programmed for periods of 30°C, 12 hr light followed by 25°C, 12 hr dark. The humidity of the rearing jars is kept near saturation by periodically adding water to the plaster-charcoal substrate. Excessive condensation is removed from the sides of the rearing jars to prevent mites from becoming trapped in the condensed water. These conditions simulate the ambient conditions of Malaysian habitats where the vectors are prevalent.

Laboratory mice, 5-7 weeks old, are used as hosts for the larval mites. Each mouse is restrained with a plastic collar and cylinder made from a snap-top plastic vial, 2.5 cm diameter, with the bottom 2/3 portion cut away. A 1.0 cm hole is bored through the center of the plastic top, and a cut made from the hole to the edge of the top. To restrain a mouse, the plastic cylinder is slipped over the mouse's head and the top quickly fitted around its neck (Fig. 7) A strip of tape secures the top to the cylinder. This device prevents the mouse from removing feeding larva with its forelegs. Chiggers are transferred from rearing jars to the ear of the host mouse by means of a moistened finely sharpened wooden applicator stick. The mouse is then placed in a cylindrical cage of hardware cloth which is positioned over a funnel apparatus until the chiggers have fed (Fig. 7). Water and food are provided the host mouse during the period of chigger feeding. The apparatus used for collecting engorged larvae consists of a 15 cm polystyrene powder funnel with a 100 ml screw cap jar, half filled with water, attached to the stem. A ring stand holds the funnel, and the mouse cage is supported over the top of the funnel by a wire frame and rubber bands. A screen wire disk placed beneath the mouse cage reduces the amount of debris falling into the jar of water. Chiggers detach from the host after feeding and drop into the jar of water beneath the funnel. The jar is then removed and its contents poured through No. 1 filter paper in a Buchner funnel mounted on a filtering flask. The larvae can then be transferred to rearing jars with a moistened finely sharpened wooden applicator stick or fine brush.

An important consideration in the rearing of the infected colonies of Leptotrombidium is that with few exceptions only female progeny have been produced (9). This requires that a normal colony of the same species as the infected one be synchronized to provide normal males or their spermatophores (10) for uptake by infected

females. Males can only be distinguished by placing individual adult mites in separate containers and subsequently examining the containers under a microscope (60X magnification) for spermatophores (Fig. 7, 8). If the adult mite is a male and has fed on collembola eggs, spermatophores are usually observed within 4 days.

Some typical results of a larval feedings are shown in Table 2. The number of mites attaching after being placed on the mouse was not determined, but fed larvae were recovered from 1 to 4 days after exposure to the host. No significant differences were apparent in percent recovery or feeding times of infected and non-infected larvae of either species. Fed larvae that had recently detached from the host were observed to crawl about in the rearing jars for a day or more before becoming quiescent. When sufficiently engorged, larvae developed to protonymphs within 3 days post feeding. The time from egg to adult using the methods described ranged from 57-92 days with an average of 74 days. Many other trombiculids, including the vector L. (L.) deliense (Walch) and the North American pest species, Eutrombicula alfreddugesi (Oudemans), have been reared by the described methods.

As stated above, recent studies (7) indicate that the mite vectors of R. tsutsugamushi are also the hosts of the agent in nature. Therefore, the rickettsiae found in mites could thus represent pristine strains that have not been modified by laboratory manipulation. Further, differences between the strains of R. tsutsugamushi carried by individual mite colonies could be of great significance both in the design of experimental vaccines and in epidemiological studies. Groups of highly susceptible inbred C3H/He Dub mice (11) were infected with either yolk sac suspensions containing 1,000 - 10,000 LD_{50} of R. tsutsugamushi strains Karp or Gilliam or by infections induced by feeding 5 infected L. fletcheri or L. arenicola larvae on the subject animals. The infected mice were then subdivided into two sub-groups. One sub-group was treated with chloramphenicol for 21 days (12) while the other sub-group was left untreated to serve as a normal control. In the untreated sub-group, all the mice inoculated with the Karp and Gilliam strains as well as those infected by the L. arenicola larva died with signs compatible with infection with R. tsutsugamushi. None of the mice infected by allowing L. fletcherii larva to engorge died as a result of the feedings. Seven days following the termination of the chloramphenicol treatment, the treated mice and a second set of controls were challenged with yolk sac suspensions containing 1,000 - 10,000 LD_{50} of either Karp or Gilliam strains of R. tsutsugamushi or by allowing 5 infected L. fletcheri or L. arenicola larvae to feed on the subject animals.

The results are given in Table 3 and show that 100% of the C3H/HeDub mice initially infected as described above with strains Karp, Gilliam, L. arenicola and + 97% of the mice vaccinated with L. fletcherii survived a challenge infection with heterologous strains of R. tsutsugamushi. Strains Karp, L. arenicola and even Gilliam (strain Gilliam is virtually innocous in Wrc:(ICR) laboratory mice) were shown to produce lethal infections in 100% of the control groups of C3H/HeDub mice. However, infection resulted in a mortality rate of only 12% in the C3H/HeDub controls challenged with the R. tsutsugamushi strain carried by L. fletcherii. Although strains Karp, L. arenicola and L. fletcheri all contain a common antigenic factor "Karp" utilized in fluorescent antibody typing procedures, this factor does not appear to be related to the virulence of the agent under examination. Previous studies (2) showed that although an experimental killed scrub typhis vaccine prepared from the Karp strain (13) protected 100% of C3H/HeDub mice against homologous intraperitoneal challenge it failed to protect any of the vaccinated mice against infection induced by feeding larval L. arenicola on the vaccinated animals. The failure of a killed vaccine prepared from one mouse lethal strain to protect mice against natural, heterologous challenge by another pristine mouse lethal strain provides one explanation for the failure of a killed vaccine prepared from strain "VOLNER" (Phillipine origin) to protect human beings exposed to endemic scrub typhus infection in Japan (14).

The above studies confirm the identity of the agents present in the L. fletcherii and L. arenicola colonies as R. tsutsugamushi; show that R. tsutsugamushi in a pristine state demonstrates strain heterogeneity long recognized in laboratory studies with the agent; and the heterogeneity demonstrated provides both an explanation for the failure of various vaccines to protect human beings and, possibly, the variations in the severity of clinical disease observed in diverse geographical locales (15). Studies are being pursued to provide further information on this interesting host-parasite relationship.

TABLE 1

Hibernating *Culex pipiens* collected at abandoned Army Forts located in Maryland, New Jersey and Delaware during the Winter of 1978

Mosquito Collection			Laboratory Handling				
				Blood fed		Non blood fed	
Location	Date Collected	Number Collected	Days held in insectary before blood-feeding	No. Pools tested	No. isolations	No. Pools tested	No. isolations
Ft. Washington, MD	3 March	379	25	9	1	29	1
Ft. Washington, MD	10 March	252	18	5	0	20	0
Ft. McHenry, MD	8 March	41	20	2	0	2	0
Ft. Mott, N.J.	13 March	965	15	78	2	19	0
Ft. Dupont, DE	14 March	83	14	6	0	2	0
TOTALS		1720		100	3	72	1

Table 2. Recovery of fed larvae and average feeding times for *Rickettsia tsutsugamushi* infected and noninfected mites using the mass feeding technique

Species and status	Total no. mice	Total no. fed Larvae/No. applied (%)		Average feeding time (days)
Leptotrombidium (L.) arenicola (infected colony)	23	81/163	(49%)	2.5
L. (L.) arenicola noninfected colony	6	15/32	(47%)	2.5
Leptotrombidium (L.) fletcheri (infected colony)	20	91/172	(53%)	2.0
L. (L.) fletcheri noninfected colony	7	25/44	(57%)	2.0

Table 3. Cross protection tests with various strains of R. tsutsugamushi

Original strain	Challenge [1]							
	Treated [2]				Control [3]			
	Karp	Gilliam	L. arenicola	L. fletcheri	Karp	Gilliam	L. arenicola	L. fletcheri
Karp strain, yolk sac suspension IP	0/10 [4]	0/10	0/10	0/11	6/6	6/6	6/6	2/7
Gilliam strain, yolk sac suspension IP	0/10	0/10	0/11	0/10	6/6	6/6	6/6	1/6
L. arenicola strain, chigger feeding	0/10	0/10	0/10	0/10	6/6	8/8	6/6	0/6
L. fletcheri strain, chigger feeding	0/12	1/12	0/10	0/10	6/6	6/6	6/6	0/6

1 Both original infection and challenge were accomplished by identical methods: Karp and Gilliam by IP inoculation, L. arenicola and L. fletcheri by allowing chiggers from infected colonies to feed to repletion.

2 All mice infected with rickettsia by chigger feeding or IP inoculation followed by treatment with chloramphenicol in the drinking water. Mice were challenged 7 days after termination of the antibiotic regimen.

3 Result of simultaneous challenge of normal mice.

4 Number of dead/total in group.

Captions

Fig. 1. Survival of Culex pipiens in Decatur Bunker at Fort Washington, Maryland during winter of 1977-78.

Fig. 2. Survival of Culex pipiens in Emory Bunker at Fort Washington, Maryland during winter of 1977-78.

Fig. 3. Map of Pocomoke Cypress Swamp showing location of Magoon and Light traps.

Fig. 4. Mean daily water temperature for Atlanticus North pool in June 1978. Water temperature measured at 4 cm depth.

Fig. 5. Abundance of Ae. atlanticus larvae in 100 m^2 study area at Atlanticus North, PCS in June 1978. Solid line represents total numbers of larvae in pool and dotted line the number of larvae per m^2 flooded.

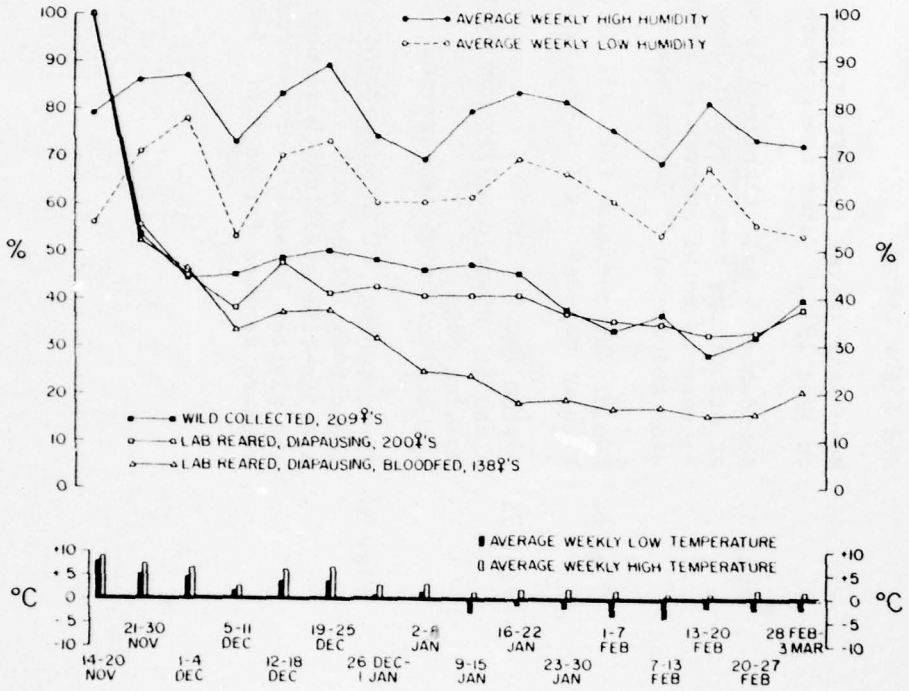
Fig. 6. Map of Chincoteague National Wildlife Refuge showing location of net and light traps.

Fig. 7. System for feeding and recovering larval mites (chiggers). A and B: plastic collar and restrained mouse; C: holding cage for infested mouse; D: funnel apparatus for collection of fed larvae.

Fig. 8. Spermatophore of L. arenicola. A white, glistening, round body filled with seminal material, bourne on a hypha-like stalk which is normally embedded in the plaster substrate of the rearing jar. (Spermatophore removed for easiter visualization 60X).

Fig. 1

SURVIVAL OF CULEX PIPIENS IN DECATOR BUNKER AT FORT WASHINGTON, MARYLAND DURING WINTER OF 1977-78



89

Fig. 2

SURVIVAL OF CULEX PIPIENS IN EMORY BUNKER AT FORT WASHINGTON, MARYLAND DURING WINTER OF 1977-78

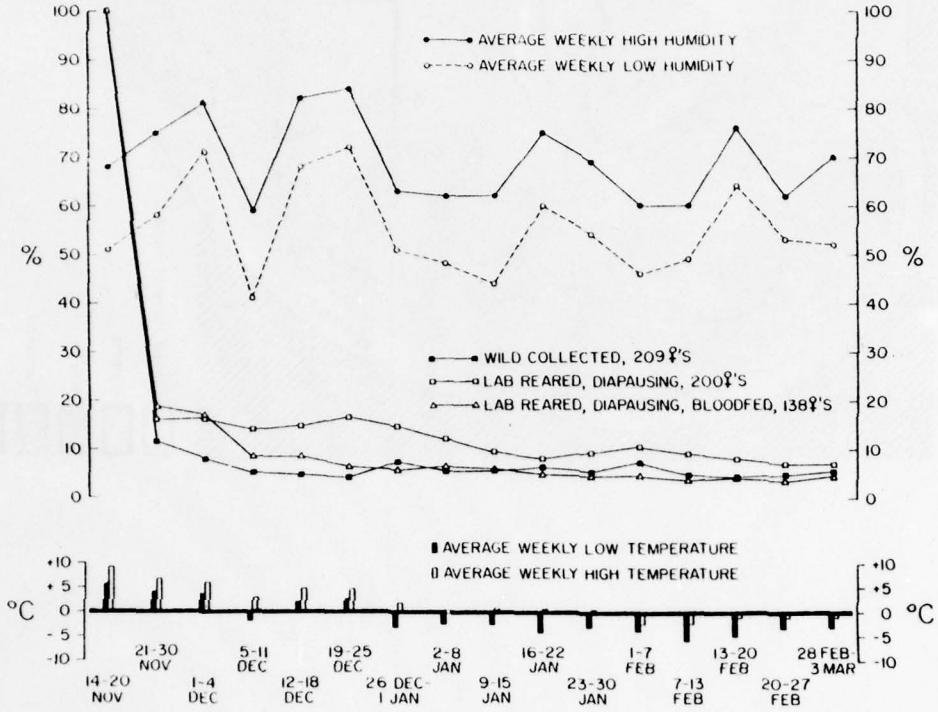


Fig. 3

MAP OF POCOMOKE CYPRESS SWAMP SHOWING LOCATION OF MAGOON AND LIGHT TRAPS

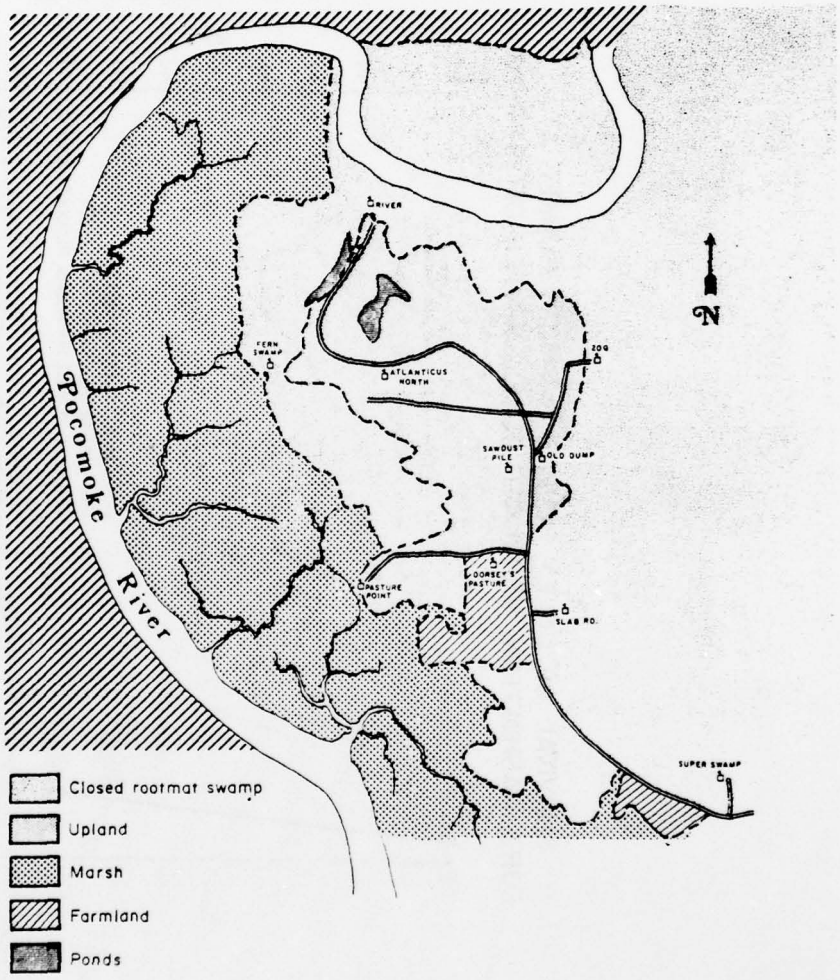


Fig. 4

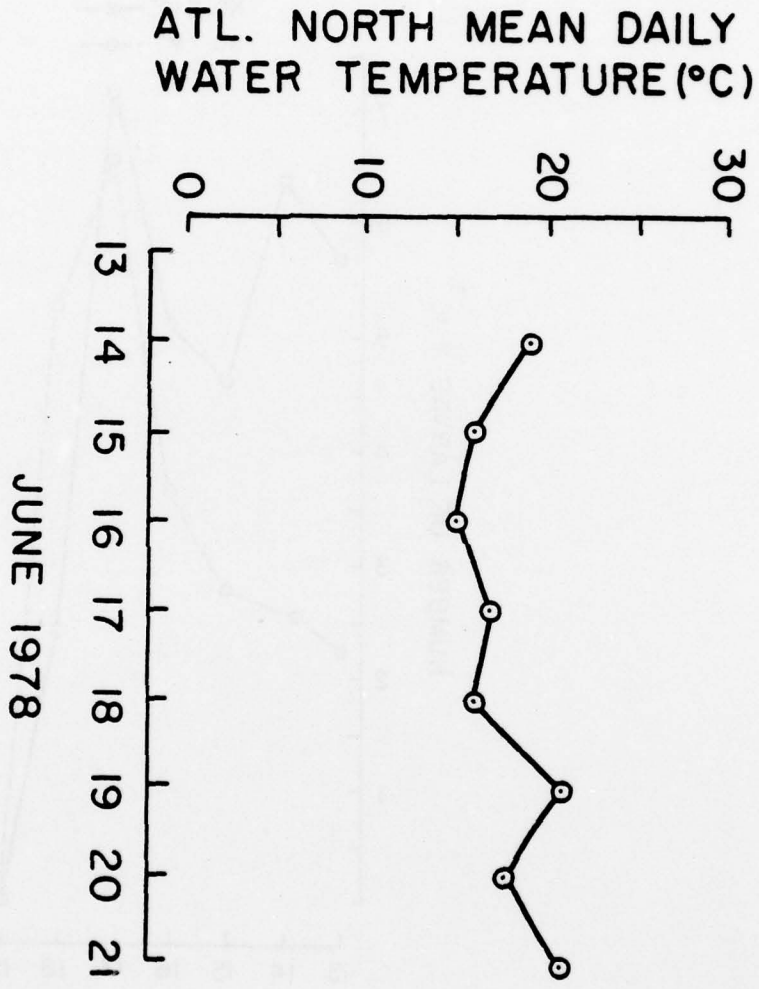

ATL. NORTH MEAN DAILY
WATER TEMPERATURE (°C)
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20
21
JUNE 1978

Fig. 5

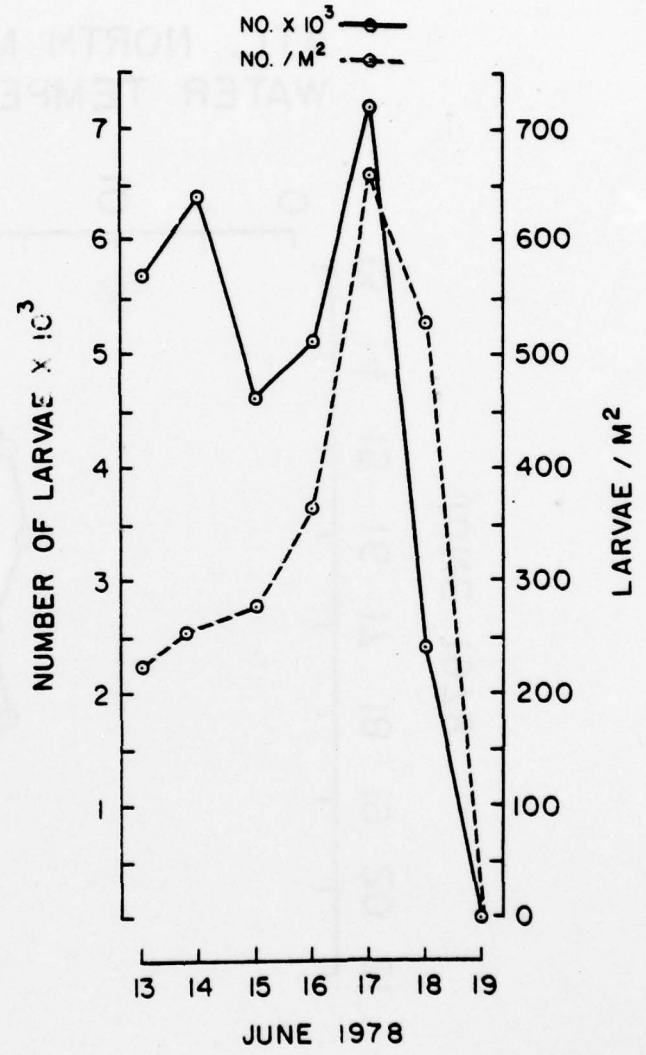
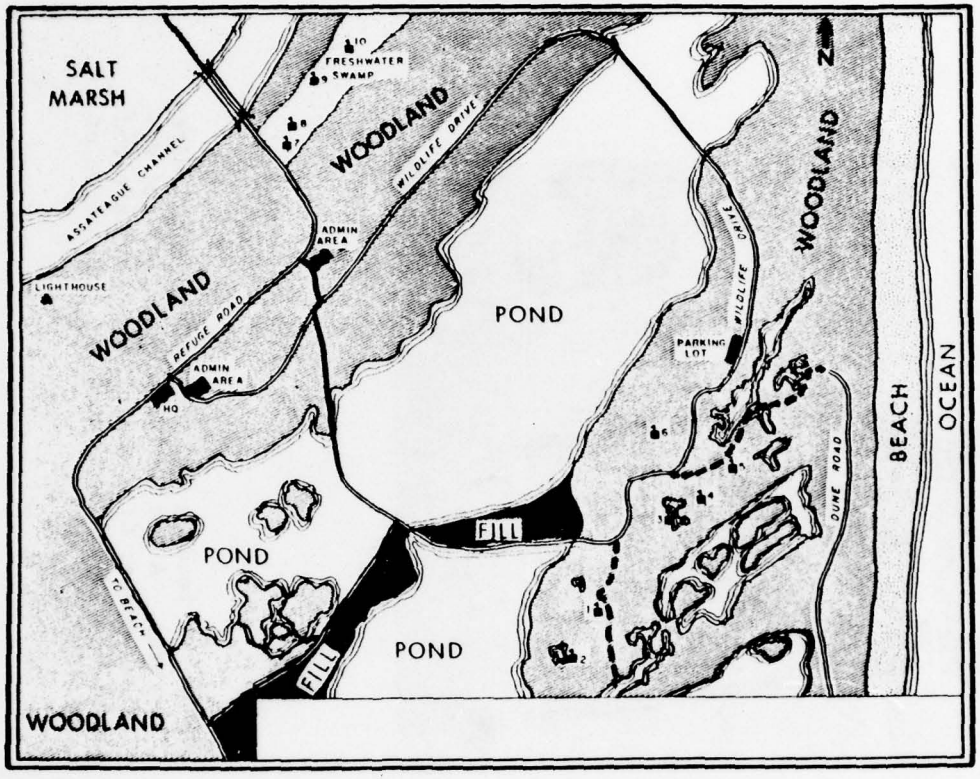

NO. X 10³
NO. / M²
NUMBER OF LARVAE X 10³
LARVAE / M²
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3
4
5
6
7
100
200
300
400
500
600
700
0
13
14
15
16
17
18
19
JUNE 1978

Fig. 6

MAP OF CHINCOTEAGUE NATIONAL WILDLIFE REFUGE SHOWING LOCATION OF NET AND LIGHT TRAPS



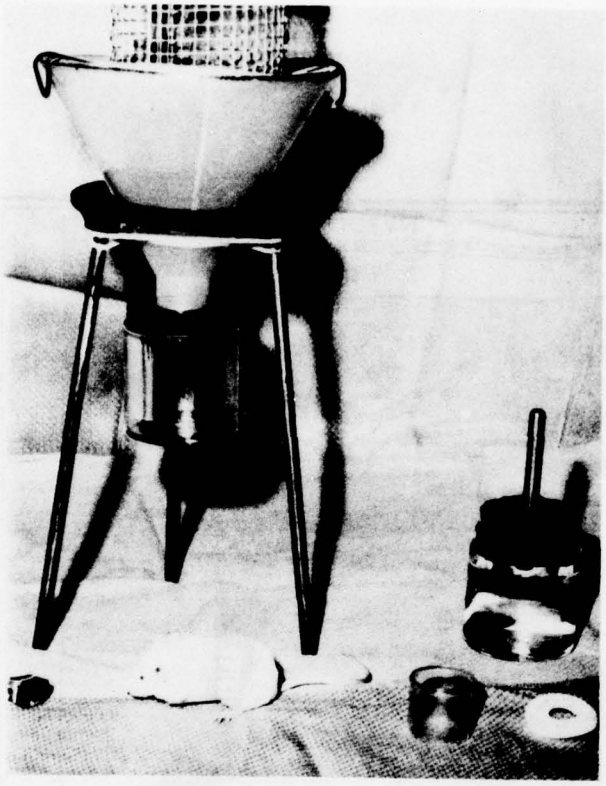


Figure 7. System for feeding and recovering larval mites (chiggers). A and B: plastic collar and restrained mouse; C: holding cage for infested mouse; D: funnel apparatus for collection of fed larvae.

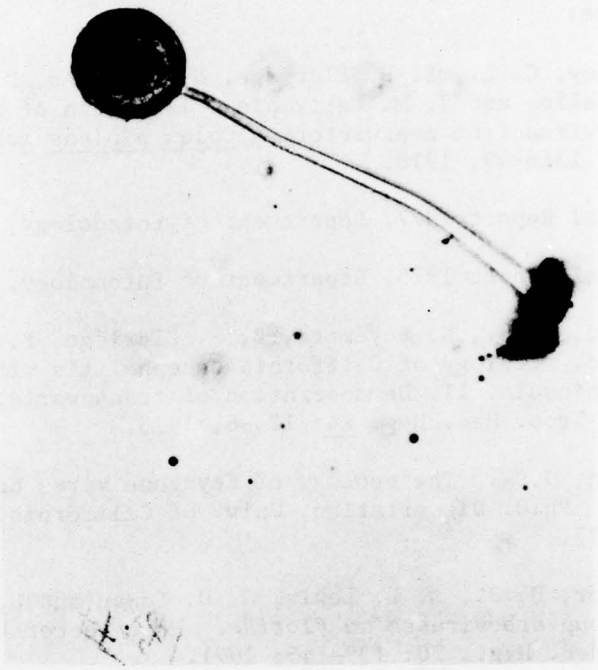


Figure 8. Spermatophore of L. arenicola. A white, glistening, round body filled with seminal material, bourne on a hypha-like stalk which is normally embedded in the plaster substrate of the rearing jar. (Spermatophore removed for easier visualization 60X).

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 121 Ecology and Control of Disease Vectors and Reservoirs

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Reference:

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1. Bailey, C. L., B. F. Eldridge, D. E. Hayes, D. M. Watts, R. F. Tammariello, and J. M. Dalrymple. Isolation of St. Louis encephalitis virus from overwintering Culex pipiens mosquitoes. Science, 199: 1346-49, 1978.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Basic Pharmacological Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	3.0	47
FISCAL YEAR CURRENT 79	3.0	137

19. RESPONSIBLE DOD ORGANIZATION

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ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: RAPMUND, COL G.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of Experimental Therapeutics
ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: HEIFFER, Dr. M. H.
TELEPHONE: 202-576-3014
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: KORTE, CPT D. W. Jr.
NAME: SCHUSTER, MAJ B.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacology; (U) Medicinals; (U) Drugs; (U) Shock; (U) Toxicity; (U) Liposomes

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Research is directed toward investigating special areas of the pharmacology of potential drugs of military importance, their interactions, their mechanisms of action, and the development, characterization and improvement of animal models for defining specific applicable parameters.

24. (U) Drugs are tested in animal models specifically designed to pinpoint mechanisms of pharmacological effects, effects on physiological responses, and effects on protozoan systems. In vitro models are being used as well.

25. (U) 77 10 - 78 09 The acute cardiovascular and respiratory activity of intravenous Pentostam in the rabbit at 80 mg antimony/kg and above was transient bradycardia, decreased arterial blood pressure, increased T wave in the heart and mild tachypnea; at large doses (greater than 320 mg antimony/kg) apnea was seen. A single injection of liposomes containing lipid A produced an immune response against lipid A equivalent to that produced by other methods of immunization and also produced an immune response against phosphocholine, phosphatidylcholine and sphingomyelin. Menoctone produced anticoagulation effects in rabbits fully antagonized by concurrent vitamin K administration. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 122 Basic pharmacological studies

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: CPT D. Korte, MAJ B. Schuster, Dr. H. Lowensohn, Dr. R. Rozman, SP5 M. Neidig

1. Description.

The basic research efforts of the department are directed towards several major areas. They are: the pharmacology of promising medicinal agents and of certain toxic substances; drug interactions with, and the nature of, adrenergic receptors; further characterization of new drug delivery techniques; and the development of new or modification of existing techniques to characterize drug effects.

Appropriate pharmacological, physiological, electrophysiological and biochemical studies are conducted both in vivo and in vitro. Many of these studies emphasize interactions of potential drugs with standard pharmacological agents. An important feature is ready access to the vast inventory of serially related and diverse chemicals which can be used in detailed studies of the nature of drug interactions with biological systems.

2. Anticoagulant effects of menoctone (WR 49,808) in the rabbit.

a. Background:

The anticoagulant effect of some 3-substituted 2-hydroxyl-1,4-naphthoquinones (Smith et al., 1946) and antagonism of that effect by vitamin K_1 (Smith, 1947) were demonstrated in rats a number of years ago. Synthesis of one member of this class, 2-hydroxy-3-(ω-cyclohexyloctyl)-1,4-naphthoquinone (menoctone, WR 49,808) (Fieser et al., 1967), was carried out to determine its potential antimalarial effect. Efficacy studies in animals demonstrated the causal prophylactic activity of this compound against Plasmodium berghei infections in mice (Berberian et al., 1968). Because of the limited number of classes of drugs with causal prophylactic properties the compound was investigated in animals in preparation for clinical trials in man. As part of these investigations the anticoagulant properties of the drug were evaluated as a potential

side effect. Reported here are the anticoagulant effects of menoctone and their full antagonism by vitamin K.

b. Materials and methods:

Twenty seven adult male albino rabbits, approximately 2-3 kg each, were divided into 4 groups and dosed as follows. Eight control (C) animals received no medication. Five rabbits (K) received aqueous colloidal vitamin K_1 intraperitoneally (ip), 1 mg/kg/day for 2 consecutive days. Eight rabbits (N) were given single oral doses of 900 mg/kg of the naphthoquinone WR 49,808 by gavage. Six animals (NK) were given single oral doses of 900 mg/kg of WR 49,808 by gavage plus concurrent vitamin K_1 ip, 1 mg/kg, with 1 mg/kg vitamin K_1 ip on the following day. Blood samples were taken by cardiac puncture into plastic syringes 48 hr after the initial dose in all groups. Aliquots of blood were treated with either sodium oxalate or sodium citrate to obtain plasma for the appropriate coagulation assays.

Standard coagulation procedures were used (Dennis and Conrad, 1968; Eichelberger, 1965). The plasma was assayed at 1 to 10 dilutions for Factor II and 1 to 5 dilutions for Factors VII, VIII, IX, X and XI. Undiluted plasma was used for determining the prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT) and the assay for Factor V.

c. Results:

Table 1 details the coagulation test results. No statistically significant differences were seen between the control group and the group which received only vitamin K_1. The group receiving WR 49,808 had a significant ($p < .05$) increase in time to clot formation when compared to controls for PT, PTT and assays for Factors II, VII, VIII, IX and X. No differences were apparent in either the TT or Factor V or Factor XI levels.

Vitamin K_1 reversed the effects of WR 49,808 in all of the assays affected by this antimalarial drug. In addition, the combination of WR 49,808 and vitamin K_1 significantly ($p < .05$) decreased time to end point of the assays for TT, Factor V and Factor XI when compared to control rabbits.

d. Discussion:

The vitamin K dependent coagulation factors are II, VII, IX and X. Their activity was depressed by WR 49,808 and this effect was reflected in the prolongation of PT and PTT. The complete reversal of these WR 49,808 related deficiencies by vitamin K indicates that this portion of the anticoagulant effect was due to the same mechanism as shown by other vitamin K antagonists. The depression of Factor VIII activity is not routinely seen during indirect anticoagulant therapy even though complete reversal by vitamin K was seen in these experiments.

That the anticoagulant effects of menoctone were reversed by concomitant administration of vitamin K was expected for those coagulation factors classically considered vitamin K dependent. The decrease in Factor VIII activity and its reversal by vitamin K was not anticipated.

Berberian et al. (1968) have shown that administration to mice of 1 mg/kg of vitamin K had no adverse effect on the antimalarial activity of menoctone against Plasmodium berghei infection. Thus the potential exists for overcoming anticoagulant toxicity of this drug without destroying the antimalarial effects.

3. Liposomes as a combined carrier and adjuvant: production of antibodies against phosphocholine, phosphatidylcholine, sphingomyelin, and lipid A by injection of liposomes containing lipid A.

a. Background:

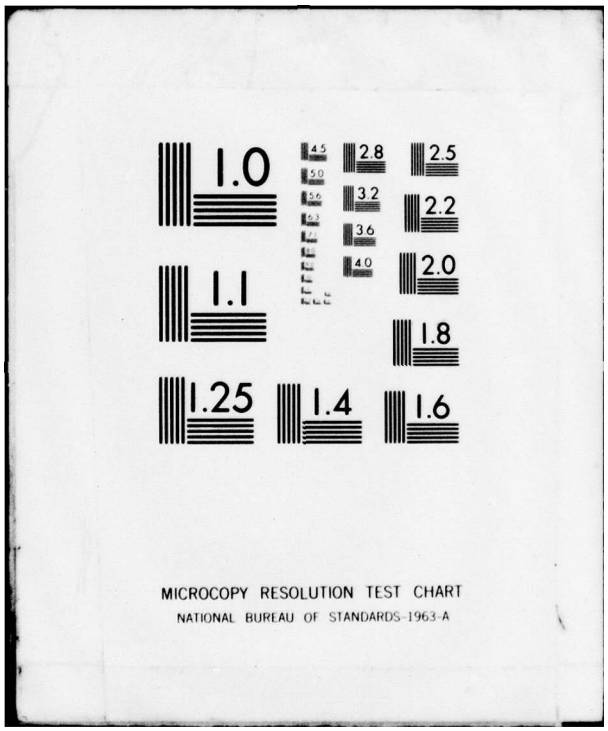
The lipid A constituent of lipopolysaccharide (LPS) is responsible for most, or all, of the endotoxic effects of LPS, including lethality, pyrogenicity, mitogenicity, and adjuvanticity (Lüderitz et al., 1973; 1968). Lipid A, as it is normally present in intact LPS, is structurally hidden from the immune system and antibodies against lipid A are not produced by immunizing with LPS (Lüderitz et al., 1973; Alving, 1977). Antibodies have been produced against purified lipid A (Alving, 1977; Galanos et al., 1971), and such antibodies react with intact LPS in liposomes or with purified lipid A in liposomes (Kataoki et al., 1971). The purpose of the present study was to investigate the use of liposomes as a carrier and adjuvant for enhancing the production of antibodies against lipid A. In the course of this study we found that injection of liposomes containing lipid A resulted in the production of antibodies that

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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMIS--ETC(U)
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MICROCOPY RESOLUTION TEST CHART
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reacted with lipid A, and also in the production of antibodies that had anti-phosphocholine specificity and reacted with phosphatidylcholine and sphingomyelin.

b. Materials and methods:

Lipids were purchased from the following sources: dimyristoyl phosphatidylcholine (DMPC) (Sigma Chemical Co., St. Louis, MO); sphingomyelin (SM) (Pierce Chemical Co., Rockford, IL); cholesterol (CHOL) (Calbiochem, La Jolla, CA); and dicetyl phosphate (DCP) (K and K Laboratories, Plainview, NY). Phosphocholine chloride (calcium salt) was purchased from Sigma. Acid treated bacterial cells (Salmonella minnesota R595), prepared by the method of Galanos et al. (1971) were kindly provided by Dr. Peter Gemski, WRAIR. Lipid A (from Shigella flexneri LPS, prepared by the Westphal method, Lot No. 509633, Difco, Detroit, MI) was produced by acetic acid hydrolysis to remove the polysaccharide chain, as described by Chang and Nowotny (1975). It contained 0.4 μmoles of lipid A. When suspended in an aqueous medium the lipid A was sonicated with a microprobe for 30 sec (output control set at 5) using a Branson Sonifier 185.

Liposomes were prepared from a mixture of phospholipid, CHOL, and DCP in molar ratios of 2/1.5/0.22. Except where indicated (in Figure 7), the phospholipid consisted of DMPC. The lipids, in chloroform, were dried together in a pear-shaped flask on a rotary evaporator, followed by one hr under high vacuum in a desiccator. A small quantity of acid-washed 0.5 mm glass beads was added to the flask, followed by the appropriate swelling solution and shaking on a Vortex mixer. The swelling solution for liposomes used for immunization or adsorption was 0.15 M NaCl, and that used in the glucose release assay for antibody activity was 0.308 M glucose. The phospholipid was 10 mM with respect to the final aqueous dispersion.

Lipid A is not soluble in water and, as ordinarily prepared, usually is not soluble in chloroform. In the past, several methods have been used to insert the lipid A into liposomes: the lipid A either was (a) included in the glucose swelling solution ("active" sensitization) (Kataoka et al., 1971), (b) treated with alkali and then included in the glucose swelling solution or added to already-formed liposomes (Kataoka et al., 1971), or (c) added in the chloroform solution with the other lipids after rendering it chloroform-soluble by treatment with dipotassium EDTA followed by Bligh-Dyer extraction (Dancey et al., 1977). Of the above methods, sensitivity to complement-

mediated immune damage was far superior using liposomes prepared by method (b) compared to method (a), and immune damage was not examined in method (c).

In this study, liposomes containing lipid A were prepared by either of two methods. Those formed by the active sensitization method (method (a) above) had the appropriate amount of lipid A sonicated in water and this was then added to an equal volume of swelling solution consisting of 0.616 M glucose. This method was used only once, where indicated (in Figure 1). In the alternative method, an aliquot of a concentrated aqueous suspension (5 mg/ml in 0.5% triethylamine) of lipid A was added to the pear-shaped flask and dried under nitrogen, using chloroform to facilitate drying. The other liposomal constituents (in chloroform) were then added to the flask and dried. The liposomes were swollen with 0.308 M glucose, or 0.15 M NaCl, as appropriate.

Male rabbits, 2.27-2.73 kg, were randomly assigned to one of the following 7 immunization schemes:

i. Lipid A (s.c.): 3 ml of lipid A (1.5 mg) in a 0.15 M NaCl suspension was mixed with 3 ml of incomplete Freund's adjuvant (IFA) and injected subcutaneously into the rear footpads, and 3 subcutaneous sites.

ii. Lipid A plus ATBC (s.c.): Acid-treated bacterial cells (S. minnesota R595) (1 mg) coated with lipid A (0.5 mg) were prepared as described by Galanos et al. (1971). This antigen was mixed with IFA and injected subcutaneously as above.

iii. Liposomes containing lipid A (s.c.): Liposomes (3 ml) containing 1.5 mg of lipid A (prepared by drying the lipid A with the other lipids) were added to an equal volume of IFA and injected subcutaneously as above. Liposomes containing lipid A are designated as follows: liposomes (+ lipid A).

iv. Lipid A (i.v.): Lipid A (1.5 mg) was suspended in 0.15 M NaCl to a total volume of 3 ml and injected intravenously into an ear vein.

v. Lipid A plus ATBC (i.v.): Lipid A (0.5 mg) and acid-treated bacterial cells (1.0 mg) were suspended in 3 ml of 0.15 M NaCl as described by Galanos et al. (1971) and were injected intravenously.

vi. Liposomes containing lipid A (i.v.): Liposomes (3 ml) containing 1.5 mg lipid A (prepared by drying the lipid A with the other lipids) were injected intravenously. Liposomes containing lipid A are designated as follows: liposomes (+ lipid A).

vii. Liposomes lacking lipid A (i.v.): Liposomes (3 ml) prepared without lipid A (designated as: liposomes (- lipid A)) were injected intravenously.

Purification of IgG antibodies was carried out. An ammonium sulfate (40% sat.) precipitate of antiserum was dialyzed against 0.0175 M sodium phosphate buffer (pH 6.5) and placed on a DEAE cellulose column. The column was eluted with the same buffer (Levy and Sober, 1960). Immunoelectrophoresis of concentrated fractions against anti-rabbit serum and anti-rabbit IgG showed one major band identified as IgG. The IgG antibodies then were purified by "affinity binding to liposomes" (Alving and Richards, 1977). In the latter procedure, liposomes lacking lipid A were used as immunoadsorbents to remove antibodies from the IgG solution. After the liposomes were washed free of unadsorbed IgG, the antibodies were recovered by elution with 1 M NaI and dialyzed against 0.15 M NaCl (Alving and Richards, 1977).

c. Results:

Glucose release from liposomes containing lipid A was investigated. Figure 1 shows complement-dependent immune damage to liposomes in which the lipid A was dried with the liposomal lipids, and to liposomes prepared by the "active" sensitization method of Kataoka et al. (1971) in which the lipid A was included in the glucose swelling solution. As shown previously by that group, this latter method required large amounts of lipid A and generally gave relatively low levels of glucose release in response to a standard amount of antiserum. In contrast, our method of drying non-alkali-treated lipid A with the lipids gave glucose release comparable to that obtained previously using alkali-treated lipid A in sphingomyelin liposomes (Kataoka et al., 1971). In all experiments described below, the lipid A, when present, was dried with the lipids because of the simplicity of the method and the high sensitivity to antibodies of liposomes produced in this way.

In our initial experiments on immunization with liposomes containing lipid A (e.g., Figure 1) we used antiserum obtained by a modification of the immunization methods of

Galanos et al. (1971) in which acid-treated bacterial cells coated with lipid A were used as the antigen, and were injected either subcutaneously or intravenously. The time course of antibody activity detected following a single injection of the Galanos-like antigen mixture in each of 3 rabbits intravenously, or 3 rabbits subcutaneously, is shown in Figure 2. The data of Figure 2 are presented for comparison with similar results observed following immunization with liposomes containing lipid A, as shown in Figure 3. In many cases, the pre-immunization bleedings contained detectable "natural" activity against lipid A, but the anti-lipid A activities were enhanced by immunization with lipid A, either alone or in liposomes (Figure 3). The activities shown in Figure 3 may be compared directly with those shown in Figure 2. Immunization with liposomes lacking lipid A did not produce antibodies against liposomes containing lipid A.

Although lipid A alone and liposomes containing lipid A both produced anti-lipid A titers, Figures 3 and 4 demonstrate that the titers clearly were higher, either with subcutaneous or with intravenous routes, when lipid A incorporated into liposomes was used as the antigen.

The investigations into immune damage to liposomes lacking lipid A showed that in every case when lipid A incorporated into liposomes was used as an antigen, antibody-like activity also appeared which resulted in complement-dependent damage to liposomes lacking lipid A. Such activity was not observed when liposomes lacking lipid A, or when lipid A in the absence of liposomes, were used as antigens. The time course of this "anti-liposome" activity in 2 rabbits injected intravenously is shown in Figure 5. In both rabbits, after 8 weeks a reimmunization (boost) resulted in a higher titer than that observed after the initial immunization. Although not shown, similar results were obtained with all 8 additional rabbits injected with liposomes containing lipid A. These observations suggested that immunization with liposomes containing lipid A resulted in production of an antibody against the liposomes themselves. To confirm this, IgG antibodies purified from rabbit number 14 antiserum 2 weeks following boost (see Materials and methods) were tested against liposomes lacking lipid A. More than 30% glucose release was observed with 200 µl of a solution of IgG antibodies that contained less than 1 µg of protein (data not shown). Adsorption of serum with liposomes lacking lipid A eliminated the titer against liposomes lacking lipid A (Figure 6).

The specificity of these antibodies was further examined by adsorption with several different preparations of liposomes lacking lipid A (Figure 7). The antibodies were adsorbed by liposomes containing only DMPC, but adsorption was enhanced by cholesterol or DCP, and the best combination contained all of these lipids. The antibodies also were adsorbed by liposomes containing sphingomyelin. Adsorption of anti-phospholipid antibodies did not influence the titer against liposomes containing lipid A (Figure 7).

Figure 8 shows that the anti-phospholipid antibodies had a specificity that was entirely inhibited by phosphocholine, and that anti-lipid A antibodies were not similarly inhibited.

d. Discussion:

We have demonstrated that liposomes containing lipid A were more immunogenic than were either lipid A alone or liposomes alone. Liposomes have been proposed previously as an "adjuvant" to enhance the immunogenicity of water-soluble antigens, such as diphtheria toxin (Allison and Gregoriadis, 1974), or human serum albumin (Van Rooijen and van Nieuwmegen, 1977), and synthetic phospholipid haptens consisting of DNP conjugated to phosphatidyl ethanolamine (Uemura et al., 1974; 1975). Lipid A, which is the mitogenically active portion of bacterial LPS, also has adjuvant properties (Lüderitz et al., 1968). Upon incorporation into liposomes, lipid A caused marked enhancement, beyond that of liposomes alone, of the immune response of synthetic DNP-phospholipid hapten. Enhanced activity against DNP-phospholipid hapten was not observed when liposomes and lipid A were injected separately (Dancey et al., 1977). In the present study, we demonstrated also that when one adjuvant (liposomes) served as the carrier for the other adjuvant (lipid A), the combination resulted in enhanced antibody formation against lipid A, and also resulted in antibody formation against the liposomes themselves.

Liposomes have been proposed as carriers for a variety of substances for pharmaceutical applications (Gregoriadis, 1976; Tyrrell et al., 1976; Poste et al., 1976). One of the major presumed advantages of liposomes is that they serve only as carriers, and that they are not ordinarily immunogenic. Even in the presence of Freund's complete adjuvant, liposomes did not evoke antibody formation or cellular immunity in guinea pigs (Uemura et al., 1974). It has even been suggested that entrapment of therapeutic enzymes in liposomes might prevent

allergic reaction to the enzymes in humans (Gregoriadis and Allison, 1974). We have confirmed that liposomes by themselves do not evoke a detectable immune response in rabbits, even in the presence of incomplete Freund's adjuvant.

The low immunogenicity of liposomes may be due to the remarkably poor immunogenicity of phosphatidylcholine, the major constituent of the liposomes. In contrast to certain other phospholipids, such as cardiolipin, phosphatidylinositol, phosphatidylglycerol, or phosphatidic acid, phosphatidylcholine is a very poor antigen (Alving, 1977). Phosphatidylcholine was even used as an auxillary lipid, together with a carrier protein (noncovalently linked methylated bovine serum albumin) to enhance the immune response against other phospholipids, but this mixture did not produce any immune response against the phosphatidylcholine (Inoue and Nojima, 1967). In the present study we demonstrated that when lipid A was incorporated into liposomes a classic immune response was produced against the phosphocholine portion of phosphatidylcholine. Anti-phosphocholine antibodies occur naturally as myeloma proteins (Leon and Young, 1971; Barstad et al., 1974) and can be raised by immunization with phosphocholine-rich substances such as pneumococcal C-teichoic acid (C-polysaccharide), or even whole pneumococci (Cozensa and Köhler, 1972; Clafin et al., 1974). Immunization with liposomes containing lipid A represents a unique method for obtaining anti-phosphocholine antibodies that might be compared with antibodies produced by other methods.

The significance of antiphospholipid antibodies is unknown. It should be noted that adsorption of anti-liposome antibodies did not lower the titer against liposomes containing lipid A (Figure 7). This observation was surprising in that the anti-phospholipid activity might have represented a high baseline that could lower the overall titer upon adsorption. It is probable that when the lipid A was present in the liposomes, binding of anti-lipid A antibodies covered the phospholipid surface, thus preventing binding of anti-phospholipid antibodies and eliminating any baseline that could be removed by adsorption.

Immunization against lipid A has been proposed as a method for protection against the clinical manifestations of endotoxic shock (Galanos et al., 1971; Rietschel et al., 1973; Rietschel and Galanos, 1977). This is based on the observation that antibodies against lipid A from LPS of one species cross-react with each of the intact LPS (endotoxins) from a broad

range of gram negative bacterial species (Galanos et al., 1971; Kataoka et al., 1971). Several reports have demonstrated a protective effect of antibodies against lipid A in animals (Rietschel et al., 1973; Rietschel and Galanos, 1977). A potential technical problem in the practical application of such protective procedures would involve the method of immunization against lipid A. Previous immunization schemes have emphasized injection of lipid A together with acid-treated bacterial cells and/or Freund's adjuvant (Galanos et al., 1971). We found (Figure 3) that lipid A by itself was immunogenic when injected intravenously, although the titers were lower than those observed by previously established immunization methods. Lipid A by itself is responsible for most of the toxic (endotoxin) properties of LPS (Lüderitz et al., 1973; 1968), and the dose that could be given for immunization in humans might be limited by such toxic effects. Immunization also might be influenced by the adjuvant, if any were used. Many adjuvants that are commonly employed in animals, such as Freund's adjuvant, cannot be used in humans. Our study shows that liposomes have promise both as a carrier and as an adjuvant for immunization against lipid A, and this combination therefore might be useful for producing protection against intact gram negative bacterial LPS.

4. The cardiorespiratory toxicity of WR 229,870 (Sodium Stibogluconate, Pentostam®) in the rabbit.

a. Background:

WR 229,870 (sodium stibogluconate, Pentostam®), a pentavalent antimony compound, is the drug of choice for visceral and cutaneous leishmaniasis (Most, 1963) as well as mucocutaneous leishmaniasis (Cahill, 1963). However, WR 229,870, which is only available for use in the United States on an experimental basis, is not always curative in the recommended treatment regimen. Consequently, the Army, which has received permission to use WR 229,870, has requested a change in the treatment regimen from a large single daily injection to multiple injections or an infusion to effect a better therapeutic response. This change in treatment regimen could also enhance the toxicity of WR 229,870. Since all antimonial compounds, regardless of valency, produce cardiac effects (Beveridge, 1963) a series of studies was conducted to define more completely the cardiorespiratory effects of WR 229,870.

Although WR 229,870 is generally well tolerated in humans, T wave alterations of the electrocardiogram have been

reported (Sapire and Silverman, 1970). We have performed preliminary studies in the anesthetized dog and rabbit. WR 229,870, equivalent to 1000 mg Sb/kg, produced only minor, transient cardiovascular alterations although no change in the electrocardiogram of the dog. However, WR 229,870 did produce a transient decrease in blood pressure, change in respiratory pattern and T wave alterations of the electrocardiogram in the rabbit following intravenous injection of doses equivalent to 40 mg Sb/kg. Thus the rabbit was chosen as a more sensitive model for assessing the cardiovascular activity of WR 229,870. The purpose of this study was to characterize the cardiorespiratory responses of the rabbit to WR 229,870 and compare the responses to a single injection with multiple injections and with an infusion of WR 229,870.

b. Materials and methods:

Seventeen male white rabbits (2.1-3.4 kg) from the Walter Reed breeding colony were anesthetized with sodium pentobarbital (30 mg/kg) given intravenously via the marginal ear vein. The left femoral vein was cannulated for injection of drug. The left common carotid artery was cannulated for measurement of arterial pressure. The trachea was cannulated for monitoring of respiratory rate via a pneumotachometer. Leads I, II and III of the electrocardiogram were recorded and heart rate derived from Lead II via an integrating cardiotachograph. All variables were recorded simultaneously on 2 Hewlett-Packard recorders which permitted concurrent monitoring of the variables at 2 different chart speeds. Temperature was monitored via a rectal thermistor probe and maintained at 37°C by use of a thermostatically controlled heating pad.

Several different dosage regimens were administered in determining the cardiovascular profile of WR 229,870. The clinical formulation of Pentostam® (Burroughs Wellcome) was used in this study. It contains Pentostam® in solution at a concentration equivalent to 100 mg Sb/ml. The rabbits were assigned to one of the following 4 dosage regimens:

Group I: The 4 rabbits in this group received a dosage of WR 229,870 equivalent to 10 mg Sb/kg followed by 20 mg Sb/kg, 40 mg Sb/kg, 80 mg Sb/kg, 160 mg Sb/kg and then 320 mg Sb/kg at 10 minute intervals. The maximum response obtained following each dose of WR 229,870 was compared with its predosing baseline value using the paired t test of significance (Zar, 1974) with a p value <0.05 considered significant.

Group II: The 4 rabbits in this group were administered the equivalent of 640 mg Sb/kg WR 229,870 over a one minute period and observed for 1 hr. Observations were made before and after the injection and every 10 min thereafter. These values were compared with the preinjection value using Dunnett's multiple comparison procedure (Zar, 1974) with a p value <0.05 considered significant.

Group III: The 5 rabbits in this group were infused with WR 229,870 at a rate equivalent to 21.3 mg Sb/kg per min for 30 min for a total dosage of 640 mg Sb/kg. The animals were then monitored for 30 min. Observations were made at 10 min intervals during the infusion and monitoring period. These values were compared with the preinfusion baseline using Dunnett's multiple comparison procedure with a p value <0.05 considered significant.

Group IV: The 4 rabbits in this group were administered 6 doses equivalent to 106.7 mg Sb/kg of WR 229,870 at 5 min intervals for a total dose of 640 mg Sb/kg and monitored for 30 min following the sixth dose. The maximum response obtained following each dose of WR 229,870 was compared with its predosing baseline value using the paired t test of significance. The maximum change from baseline for each injection was compared using Dunnett's multiple comparison procedure. In all tests a p value <0.05 was considered significant.

c. Results:

Cumulative dose response studies in the pentobarbital-anesthetized rabbits indicate that WR 229,870 produced a slight but consistent decrease in heart rate at doses equivalent to 10 mg Sb/kg (Table 2). However, this response does not appear to be dose-related until doses equivalent to 80 mg Sb/kg were reached. The responses of the lower doses may have resulted from the mechanical stimulation caused by the injection and flush. Changes in the electrocardiogram were observed beginning with the 40 mg Sb/kg dose of WR 229,870. These were most prominent in Leads II and III and consisted of an increased amplitude of the T wave and, to a lesser degree, the QRS complex plus an apparent prolongation of the QT interval with resultant loss of the P wave as a separate entity. These changes were fleeting (only 5 sec after 40 mg Sb/kg) but the duration appeared to be dose-related as this period of electrocardiogram alterations persisted for 1 min after the 320 mg Sb/kg dosing. WR 229,870 also produced a dose-related hypotensive response beginning with the dosage equivalent to 80 mg Sb/kg (Table 3). WR 229,870

equivalent to 320 mg Sb/kg produced a drop in mean arterial pressure to 60% of baseline. The effect of WR 229,870 on respiration was negligible prior to the dose equivalent to 80 mg Sb/kg (Table 4). The primary response appeared to be an increase in respiratory rate but a decrease in respiratory rate was also observed. The effect on mean arterial pressure, heart rate, respiratory rate and electrocardiogram were transient; there was no difference in the baseline values at 10 min intervals prior to each injection.

Administration as a one min injection of WR 229,870 equivalent to 640 mg Sb/kg produced marked cardiorespiratory effects (Table 5). Two of 4 animals that received this dosage regimen became apneic following the injection and did not recover spontaneous respiration for 15 and 25 min during which time they were placed on a respirator. The cardiorespiratory effects of this potentially lethal dose were hypotension, alteration in electrocardiogram and a decreased heart rate in addition to the variable respiratory effects. A dose of WR 229,870 produced a decrease in blood pressure to 46% of baseline values immediately upon completion of the injection. The blood pressure had returned to 73% of baseline by 10 min and slowly returned toward baseline value during the remainder of the 60 min observation period. Although differences in the heart rate responses were not statistically significant, WR 229,870 did produce a decrease in heart rate of 51.3 bpm (range 30-85). The electrocardiographic alterations due to 640 mg Sb/kg were similar to those observed with lower dosages. However, the duration of these responses were considerably prolonged (up to 5 min). In the rabbit with a heart rate decrease of 85 bpm there was a short burst of ectopic pacemaker activity. One rabbit also exhibited an inverted T wave after the characteristic alterations produced by WR 229,870. However, this animal was apneic and had been placed on a respirator. The respiratory responses were varied. One rabbit exhibited a brief period of bradypnea while another had a brief period of apnea. Both rabbits' respiratory rates had returned to normal within 10 min. The remaining 2 rabbits had to be placed on a respirator as they were apneic. One rabbit had a brief period of tachypnea followed by 25 min of apnea. After 25 min the animal was breathing spontaneously and had returned to baseline values after 40 min. The fourth rabbit became apneic following the WR 229,870 injection and was artificially respired for 15 min before spontaneous breathing returned. His respiration had returned to baseline values 20 min after the injection.

The cardiorespiratory effects of the equivalent of 640 mg Sb/kg WR 229,870 were attenuated by infusion over 30 min in 4 of 5 rabbits (Table 6). The fifth animal exhibited a bradypnea toward the end of the 30 min infusion and became apneic post-infusion. The animal was not placed on a respirator and after 40 min of the study was severely hypotensive with bradycardia. There were no obvious changes in the electrocardiogram except in one animal which appeared to have an increased T wave amplitude which returned to normal within one min of terminating the infusion.

The equivalent of 640 mg Sb/kg WR 229,870 was also given in 6 equal doses (106.7 mg Sb/kg each) at 5 min intervals over a 30 min period. Analysis of the mean arterial pressure, heart rate and respiratory rate data (Table 7) at 5 min following the second, fourth and sixth injections and every 10 min for the remainder of the one hr experimental period produced results similar to the 30 min infusion. Dividing the dose also attenuated the cardiorespiratory responses in 3 of 4 animals. The fourth rabbit, which exhibited a severe tachypnea characterized by shallow panting after the fourth injection of WR 229,870, became apneic and died after the fifth injection. The maximum change produced by each injection of the equivalent of 106.7 mg Sb/kg WR 229,870 are given in Table 8. This dose of WR 229,870, as expected from the dose-response studies, produced a decrease in blood pressure and heart rate and a variable response on respiration. The characteristic alterations in the electrocardiogram lasted for a period of approximately 15 sec. Cumulative administration did not modify these responses.

d. Discussion:

The unanesthetized rabbit tolerated doses of sodium antimony gluconate (Solustibosan®, a less concentrated formulation than WR 229,870) of 150 mg Sb/kg by intravenous injection while doses up to 370 mg Sb/kg caused only transient signs of collapse and doses of 400 mg Sb/kg were lethal (Weese, 1937). These data parallel our results with WR 229,870 in the anesthetized rabbit. Single doses of WR 229,870 up to 320 mg Sb/kg produced only transient effects in anesthetized rabbits while doses of 640 mg Sb/kg caused prolonged apnea in 2 of 4 rabbits following a one min injection which would have been lethal without artificial respiration.

Doses of WR 229,870, equivalent to 80 mg Sb/kg, produced a decrease in mean arterial pressure with no reflex tachycardia.

Indeed, WR 229,870 actually decreased the heart rate. A concurrent decrease in mean arterial pressure and heart rate may be due to stimulation of the Bezold-Jarisch reflex. Vagotomy, which eliminates the Bezold-Jarisch reflex, was not performed in these animals. The respiratory responses of the anesthetized rabbits to WR 229,870 injections were generally an increased rate at the lower doses and a depression at the higher doses (640 mg Sb/kg). WR 229,870 may have a biphasic action directly on the respiratory center or the response may include both a direct action to depress respiration and an indirect response of the rabbit to the hypotension which would initiate a reflex tachypnea. Additional studies are planned in unanesthetized rabbits to aid in defining the respiratory effects of WR 229,870 injection.

The characteristic changes in the electrocardiogram reported clinically following toxic doses of sodium antimony gluconate are T wave depression, variable ST segment changes and an increase in ST interval (especially in Leads II and III) which persist up to 2 weeks after discontinuation of dosing (Sapire and Silverman, 1970). Prominent changes in the electrocardiogram were also observed in the anesthetized rabbit given WR 229,870 and included increased amplitude of the T wave and QRS complex and prolongation of the ST interval. These changes were transient as they lasted for only 5 sec after doses of 40 mg Sb/kg. However, there was a dose-response relationship as increased doses of WR 229,870 prolonged the duration of the electrocardiographic alterations. Electrocardiographic analysis of the anesthetized rabbit is complicated by a baseline heart rate of 300 bpm. The ST segment is essentially nonexistent and any increase in QT interval merges the T and P waves thereby interfering with analysis of the QT interval. The increased amplitude of the QRS complex (actually an increase in R wave amplitude) may be attributable to the large volumes being administered to the rabbits dilating the ventricles and/or to the decrease in heart rate produced by WR 229,870. The slower heart rates ($\sim$225 bpm) would permit fuller excursion of the recording pens thus permitting a more accurate representation of the true amplitude of the R wave. The appropriatenesses of the rabbit as a model of the cardiotoxicity produced by WR 229,870 remains unclear. However, WR 229,870 alters ventricular repolarization experimentally in the rabbit model as well as clinically. The differences in the time course of these T wave alterations may be a function of the acute dosing schedule for the anesthetized rabbits. Additional studies are planned to include chronic dosing of unanesthetized rabbits.

The purpose of this study, in addition to characterizing the cardiorespiratory effects of WR 229,870, was to compare a single large injection of WR 229,870 with the same dose of WR 229,870 administered as an infusion or as multiple doses. A total dose of 640 mg Sb/kg WR 229,870 was chosen as it produced significant cardiovascular and respiratory effects. Injection of this dosage over a one min period caused a decrease in mean arterial pressure and heart rate and changes in the electrocardiogram similar to that produced by lower doses of WR 229,870 although more severe and prolonged. Unlike lower doses, the injection of the 640 mg Sb/kg dose of WR 229,870 depressed respiration and may even be considered lethal since 2 of the 4 rabbits had to be placed on the respirator for periods up to 25 min before spontaneous breathing recurred. The acute cardiovascular effects of WR 229,870 were eliminated by infusion or multiple injection over a 30 min period. However, these regimens were still lethal as one rabbit in each group became apneic and died. These studies indicate that the cardiovascular responses to WR 229,870 injection may be attenuated with the proper dosing regimen. However, the respiratory depression produced by this dose of WR 229,870 was not eliminated in all animals by changing the dosing regimen. The resultant lethality was a function of the dose of WR 229,870 administered and not necessarily a failure of the alternate dosing regimens to attenuate the respiratory depression. The respiratory depression produced by WR 229,870 does not represent a clinical liability because one needs massive doses of WR 229,870 to produce it and temporary supportive measures (positive pressure respiration) for 15 to 25 min enabled the animal to recover and breathe spontaneously.

Table 1

Comparison of Effects of Treatment on Coagulation Tests in Rabbits[a]

Group	Coagulation Test or Factor[b,c]									
	PT	PTT	TT	II	V	VII	VIII	IX	X	XI
Untreated Control	7.2 ± .32	23.8 ± 1.22	16.9 ± .94	11.8 ± .32	11.3 ± .17	10.2 ± .20	40.1 ± .78	35.8 ± 2.12	14.5 ± .28	46.5 ± 1.23
Vitamin K Control	7.3 ± .17	26.1 ± 1.07	15.3 ± .60	13.8 ± .55	10.4 ± .52	11.7 ± .46	43.4 ± .84	45.1 ± 1.61	14.1 ± .33	46.7 ± .77
WR 49,808	14.6[d] ± .99	50.6[d] ± 2.25	16.8 ± 1.14	15.6[d] ± .41	12.1 ± .60	34.4[d] ± 3.64	49.7[d] ± 2.01	76.6[d] ± 1.72	25.4[d] ± 1.23	43.3 ± 1.45
WR 49,808 + Vitamin K	7.3 ± .60	26.3 ± 1.90	12.1[d] ± .64	10.5 ± .49	8.3[d] ± .72	11.2 ± 1.37	36.8 ± 1.67	37.9 ± 1.60	14.9 ± .72	28.1[d] ± 1.06

[a]Time to endpoint is in seconds.

[b]Abbreviations are in text.

[c]Values are mean ± SE of the mean.

[d]Significantly different from untreated controls ($p < 0.05$).

Table 2

The Effect of Bolus Intravenous Injections of WR 229,870 on the Heart Rate of Four Pentobarbital-Anesthetized Rabbits[a]

Dose (equiv. of Sb)	Baseline	Post-Injection
10 mg/kg	297.5 ± 6.3	285.0*[b] ± 6.5
20 mg/kg	302.5 ± 8.5	290.0* ± 7.1
40 mg/kg	300.0 ± 10.8	287.5* ± 9.5
80 mg/kg	290.0 ± 12.9	272.5* ± 13.2
160 mg/kg	285.0 ± 11.9	262.5* ± 9.5
320 mg/kg	292.5 ± 7.5	267.5* ± 7.5

[a]Values are mean ± SEM in beats per minute.

[b]The asterisk indicates that the values are significantly different from baseline (paired t test, $p < 0.05$).

Table 3

The Effect of Bolus Intravenous Injections of WR 229,870 on the Mean Arterial Pressure of Four Pentobarbital-Anesthetized Rabbits[a]

Dose (equiv. of Sb)	Baseline	Post-Injection
10 mg/kg	87.5 ± 4.3	86.3 ± 2.4
20 mg/kg	87.5 ± 4.3	81.3 ± 3.8
40 mg/kg	85.0 ± 4.1	83.8 ± 3.8
80 mg/kg	82.5 ± 2.5	72.5*[b] ± 1.4
160 mg/kg	80.0 ± 2.9	60.0* ± 3.5
320 mg/kg	83.8 ± 3.8	50.0* ± 7.9

[a]Values are mean ± SEM in mmHg.

[b]The asterisk indicates that the values are significantly different from baseline (paired t test, $p < 0.05$).

Table 4

The Effect of Bolus Intravenous Injections of WR 229,870 on the Respiratory Rate of Four Pentobarbital-Anesthetized Rabbits[a]

Dose (equiv. of Sb)	Baseline	Post-Injection
10 mg/kg	45.0 ± 4.4	48.0 ± 4.4
20 mg/kg	46.8 ± 2.1	45.8 ± 2.6
40 mg/kg	43.5 ± 2.9	42.8 ± 2.6
80 mg/kg	42.0 ± 3.7	47.5 ± 10.3
160 mg/kg	39.8 ± 3.8	48.5 ± 7.4
320 mg/kg	42.0 ± 4.4	65.8 ± 18.1

[a]Values are mean ± SEM in respirations per minute.

Table 5

The Mean Arterial Pressure, Heart Rate and Respiratory Rate Responses of Anesthetized Rabbits to Injection of the Equivalent of 640 mg Sb/kg WR 229,870 Over a One Minute Period[a]

	Baseline Value	Time in Minutes After WR 229,870 Injection						
		1	10	20	30	40	50	60
Mean Arterial Pressure (mmHg)	70.0 ± 3.5	32.5[b] ± 9.3	51.3 ± 9.4	50.0 ± 4.1	55.0 ± 5.4	57.5 ± 4.3	56.3 ± 4.3	60.0 ± 7.1
Heart Rate (bpm)	278.8 ± 19.6	227.5 ± 8.5	297.5 ± 20.6	307.5 ± 28.7	310.0 ± 28.9	310.0 ± 21.6	300.0 ± 13.5	307.5 ± 14.4
Respiratory Rate (#/min)	30.0 ± 8.8	13.0[c] ± 11.1	19.8[d] ± 14.2	30.0[e] ± 13.6	34.5 ± 10.0	33.5 ± 9.3	35.0 ± 13.6	29.0 ± 11.2

[a]Values are mean ± SEM for 4 rabbits.

[b]Significantly different from baseline at the 5% level of significance (Dunnett's multiple comparison procedure).

[c]Two of 4 rabbits were apneic. Their respiratory rate was considered to be zero.

[d]Two of 4 rabbits were on respirator because apneic. Their respiratory rate was considered to be zero.

[e]One of 4 rabbits was on respirator because apneic. Its respiratory rate was considered to be zero.

Table 6

The Mean Arterial Pressure, Heart Rate and Respiratory Rate Responses of Anesthetized Rabbits to Infusion of the Equivalent of 640 mg Sb/kg WR 229,870 Over a 30 Minute Period[a]

	Baseline Value	WR 229,870 Infusion (Time-Minutes) 10	20	30	Observation Period (Time-Minutes) 40	50	60
Mean Arterial Pressure (mmHg)	80.0 ± 3.5 (75)	65.0 ± 6.5 (65)	60.0 ± 5.0 (60)	60.0 ± 5.0 (55)	61.3 ± 5.2 (25)	65.0 ± 5.0	66.3 ± 6.9
Heart Rate (bpm)	292.5 ± 18.0 (290)	272.5 ± 22.5 (260)	265.0 ± 25.0 (260)	262.5 ± 22.9 (260)	267.5 ± 21.0 (150)	280.0 ± 17.8	275.0 ± 17.6
Respiratory Rate (#/min)	38.5 ± 4.2 (40)	41.3 ± 10.6 (65)	43.5 ± 5.8 (64)	44.3 ± 2.2 (22)	39.0 ± 2.4 (0)	38.5 ± 1.3	39.0 ± 6.4

[a]Values are mean ± SEM for 4 rabbits.

[b]Values in parentheses are for a fifth rabbit that died from anoxia.

Table 7

The Mean Arterial Pressure, Heart Rate and Respiratory Rate Responses of Anesthetized Rabbits to a Divided Dosage Regimen Equivalent to 640 mg Sb/kg of WR 229,870 Administered Over a 30 Minute Period[a,b]

	Baseline Value	WR 229,870 Injection Period (Time-Minutes) 10	20	30	Observation Period (Time-Minutes) 40	50	60
Mean Arterial Pressure (mmHg)	71.7 ± 1.7 (65)[c]	66.7 ± 3.3 (50)	60.0 ± 5.8 (30)	58.3 ± 7.3	56.7 ± 4.4	55.0 ± 0.0	55.0 ± 0.0
Heart Rate (bpm)	290.0 ± 15.3 (260)	280.0 ± 5.8 (220)	280.0 ± 11.6 (250)	280.0 ± 11.6	276.7 ± 14.5	273.3 ± 12.0	273.3 ± 12.0
Respiratory Rate (#/min)	42.0 ± 7.6 (34)	45.3 ± 10.7 (38)	41.3 ± 15.5 (85)	38.7 ± 16.7	46.0 ± 18.0	44.7 ± 13.1	50.0 ± 5.8

[a]Rabbits were administered 6 doses of WR 229,870 equivalent to 106.7 mg Sb/kg each at 5 minute intervals.

[b]Values represent mean ± SEM for 3 rabbits.

[c]Values in parentheses are from a fourth rabbit that died from anoxia.

Table 8

Comparison of the Mean Arterial Pressure, Heart Rate and Respiratory Rate Changes Produced by Six Injections of WR 229,870 Equivalent to 106.7 mg Sb/kg Each Administered to Anesthetized Rabbits[a]

	Injections of WR 229,870					
	1/4[b]	2/4	3/4	4/4	5/4	6/3[b]
Mean Arterial Pressure (mmHg)	-12.5[c] ± 3.2	-13.8[c] ± 3.1	-16.3[c] ± 3.1	-12.5[c] ± 3.2	-12.5[c] ± 2.6	-13.3[c] ± 3.3
Heart Rate (bpm)	-12.5[c] ± 2.5	-12.5[c] ± 2.5	-12.5[c] ± 2.5	-12.5 ± 6.3	-12.5 ± 6.3	-16.7 ± 6.7
Respiratory Rate (#/min)	4.0 ± 4.2	-1.0 ± 5.0	2.5 ± 3.9	-6.8 ± 6.8	-20.8 ± 23.6	-13.3 ± 7.7

[a]Values represent mean change from baseline ± SEM of injections of WR 229,870 equivalent to 106.7 mg Sb/kg each (total dose = 640 mg Sb/kg).

[b]Four rabbits were dosed. Three survived all injections while the fourth died of apnea immediately after the fifth injection (see text for details).

[c]Significantly different from baseline at 5% level of significance (paired t test of significance).

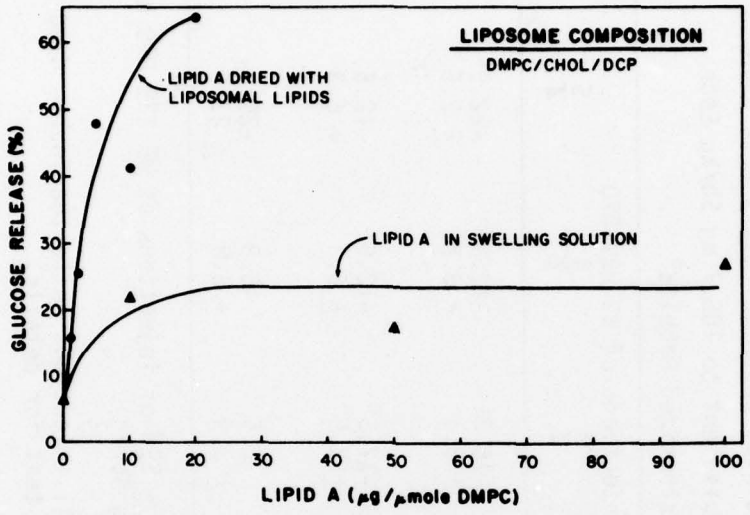


Figure 1. Immune sensitivity of two preparations of liposomes containing lipid A. The liposomes used in the upper curve (●-●) were prepared by drying the lipid A and the other lipid constituents together prior to swelling. The liposomes used in the lower curve (▲-▲) were formed by adding the lipid A to the swelling solution (see Methods for further details). Glucose release was measured in the presence of 50 μl of anti-lipid A serum from a rabbit immunized by scheme ii (see Methods).

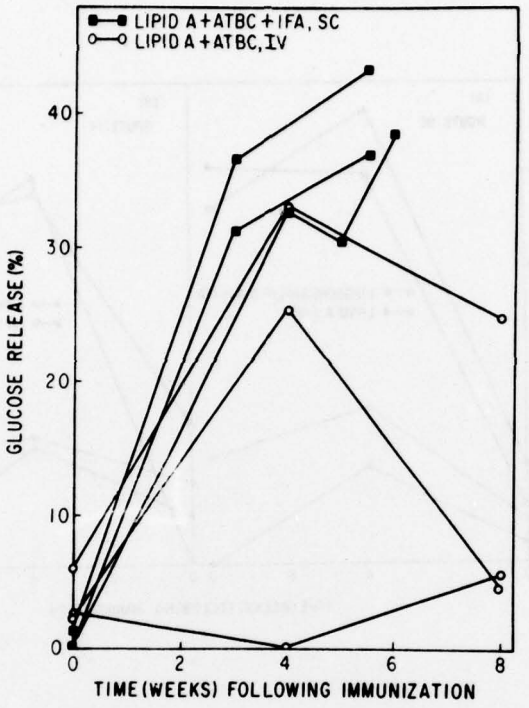


Figure 2. The time course of antibody activity against lipid A following a single injection of acid-treated bacterial cells coated with lipid A. The antigen mixture consisted of acid-treated bacterial cells coated with lipid A using scheme ii or v (see Methods), as appropriate. Each point represents complement-dependent glucose release from liposomes containing 20 µg lipid A per µmole of DMPC, and using 15 µl of antiserum. Each line represents a separate rabbit.

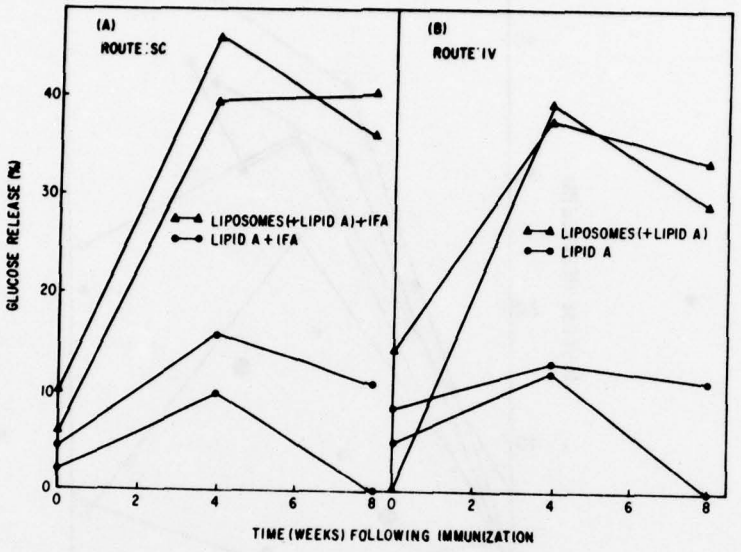


Figure 3. Time course of anti-lipid A activity following injection of liposomes containing lipid A, or of lipid A alone. Rabbits were immunized by scheme i, iii, iv, or vi (see Methods), as appropriate. Each point represents complement-dependent glucose release from liposomes containing 20 μg of lipid A per μmole of DMPC, and using 15 μl of antiserum. Each line represents a separate rabbit. Glucose release from liposomes lacking lipid A is not shown because separate experiments (cf. Figure 7 and Discussion) demonstrated that binding of anti-lipid A antibodies inhibited the binding of anti-phospholipid antibodies.

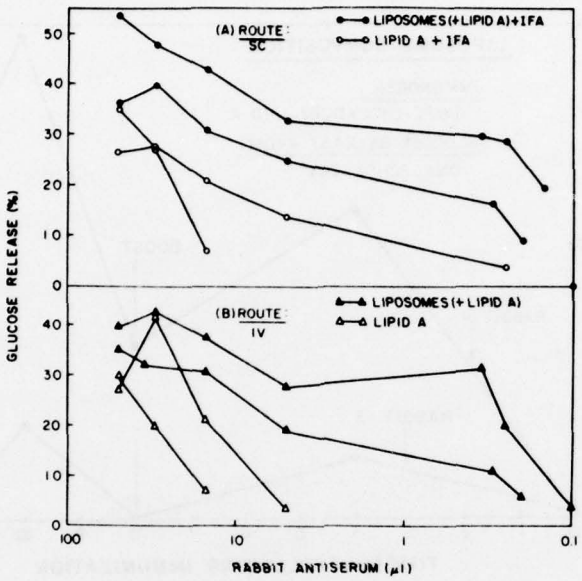


Figure 4. Titers of antisera following immunization with liposomes containing lipid A or with lipid A alone. The antisera were the same as those shown in Figure 3 and were obtained 4 weeks after initial immunization. The liposomes used for glucose release contained 60 µg of lipid A per µmole of DMPC. Each line represents a separate rabbit.

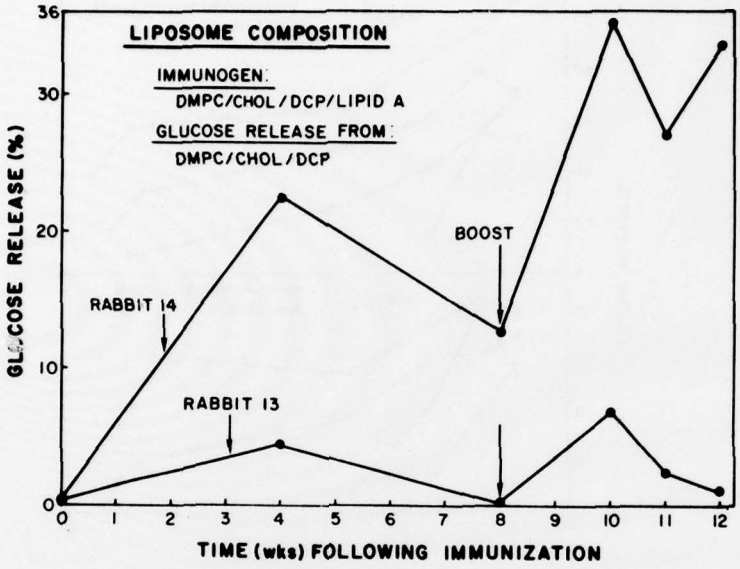


Figure 5. Complement-mediated immune damage against liposomes lacking lipid A following immunization with liposomes containing lipid A. The liposomes used for glucose release contained 20 μg of lipid A per μmole of DMPC. Antisera (15 μl) were obtained from two rabbits immunized by scheme vi (see Methods).

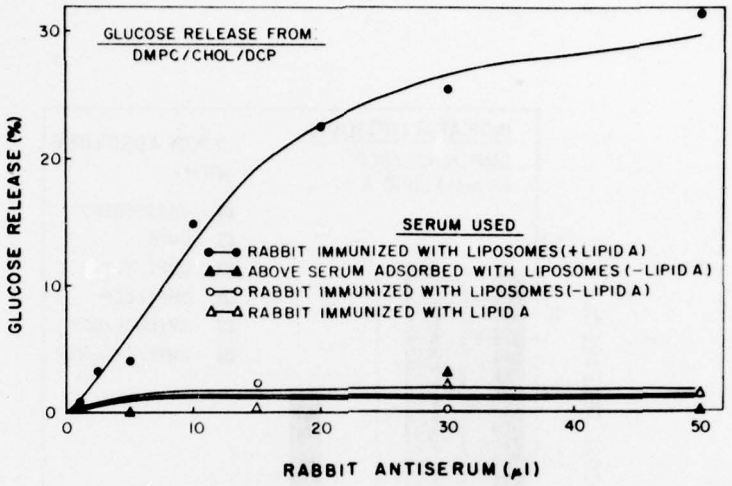


Figure 6. Absorption of activity against liposomes lacking lipid A. Antiserum (1 ml) from rabbit 14 four weeks after immunization (see Figure 5) was incubated for 30 minutes at room temperature with 0.4 ml of liposomes lacking lipid A. The liposomes were removed by centrifugation (27,000 x g, 10 min). Glucose release was measured from liposomes lacking lipid A. Activity of an equal amount of unadsorbed serum obtained following immunization of two other rabbits with either lipid A alone (i.v.) or liposomes lacking lipid A (i.v.) is also shown.

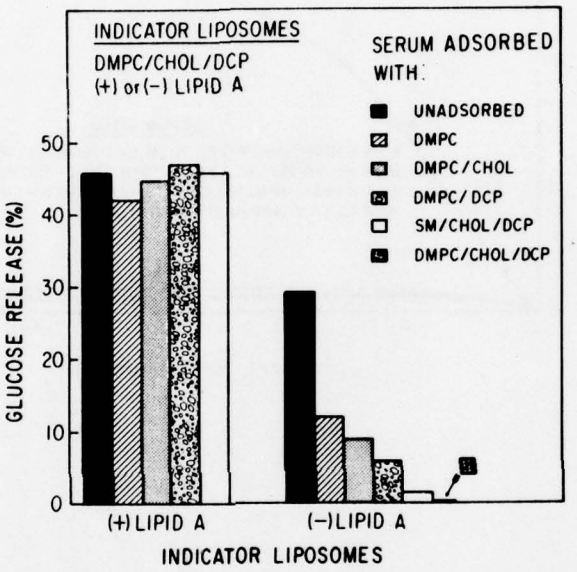


Figure 7. Effects of adsorption by liposomes lacking lipid A. Aliquots of antisera were adsorbed with the indicated liposomes as described in the legend of Figure 6. Glucose release was measured from liposomes consisting of DMPC/CHOL/DCP and containing lipid A (20 μg per μmole of phospholipid) or lacking lipid A.

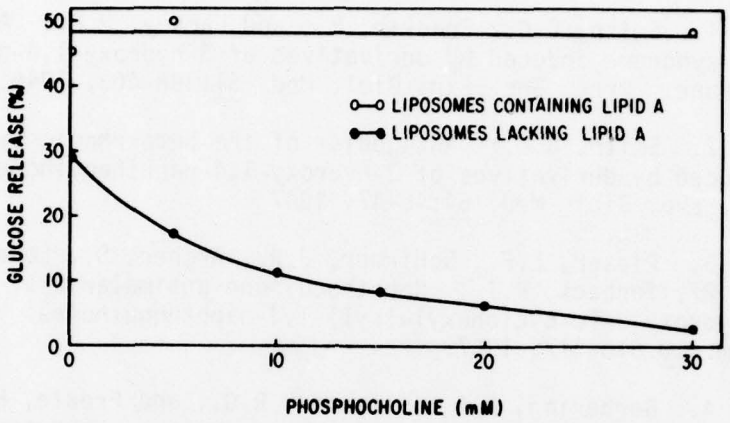


Figure 8. Inhibition by phosphocholine. Glucose release from liposomes lacking lipid A or containing lipid A (20 μg per μmole DMPC) was measured in the presence of 50 μl of antiserum from rabbit 14 four weeks after immunization (see Figure 5). Phosphocholine was added to the assay cuvette prior to the addition of complement.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 122 Basic pharmacological studies

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6445	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	123
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114 F			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Research on Cellular Injury

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003000 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	6	340
	CURRENT 79	6	379

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: RICHARDSON, EARL C.

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Cyclic Nucleotides (U) Antigen (U) Drug Resistance (U) Cell Membranes

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of this work unit is to define the biochemical parameters which may monitor or signal cell injury, alteration or regeneration. The definition of such changes will lead to enhanced understanding of the mechanisms involved in the healing of combat wounds and trauma, in tissue regeneration and in cellular drug resistance.

24. (U) The biochemical processes which determine and accompany lymphocyte transformation during trauma and rejection will be studied. Lymphocytes in single and mixed culture are used as model systems and the relation between cyclic nucleotide changes, macro molecular synthesis and antibody production are determined. The influence of drugs on parasitized cells or bacteria are investigated to define biochemical parameters related to sensitivity or resistance, particulary transport systems and molocules controlling the uptake of the drug. The serum, urinary and bone marrow levels of metabolites or enzymes are determined and related to cell trauma, death, regeneration, or proliferation.

25. (U) 77 10 - 78 09 Polyamine secretion in the urine during cellular changes varies with respect to both quantity and types. A reduction in the level of cyclic nucleotide has been found to occur in allogenic compared to syngenic lymphocyte cultures. The drug mefloquine is a potent bactericidal agent which suppresses macromolecular synthesis, destroy permeability barriers, and induces lysis of cells. The amino acid composition of a prostatic acid phosphatase has been determined in part. For technical reports see WRAIR Annual Progress Report 1 Oct 77 to 30 Sep 78.

*Available to contractors upon originator's approval.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 123 Biochemical Research on Cellular Injury

Investigators
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The objective of this work unit is the definition of the biochemical parameters which may indicate or monitor cell injury, alteration, or proliferation. Cellular injury may result from combat wound trauma or infection, cellular alteration may occur upon antigen challenge or result in drug resistance, and proliferation of cells may be associated with wound healing, graft rejection, or pathological states. The following studies were conducted:

1. Studies on the mode of action and mechanism of resistance to antimalarial drugs.

2. Investigations on the immune response.

3. Correlation of a cellular enzyme with organ pathology and treatement.

4. Determination of metabolite changes as evidenced by alteration in their levels in body fluids and their secretion.

1. Studies on the mode of action of antimalarial drugs. Possible mode of action of Mefloquine.

Mefloquine is an antimalarial drug, developed to provide effective chemotheraphy against plasmodia resistant to chloroquine and its congeners. Structurally, mefloquine is a member of the quinolinemethanol series of drugs of which quinine is the prototype. Both quinine and mefloquine cause the loss of plasmodial viability and in so doing induce the disappearance of haemozoin granules, and of pigment aggregated by prior exposure to chloroquine (Peters 1977), suggesting that their modes of action are similar if unknown. However, very few mode of action studies have been carried out with mefloquine, and these (Davidson et al, 1975; Peters et al, 1977) indicate only that mefloquine does not bind to DNA. This observation is in apparent contrast to observations on quinine

which has been found to bind to DNA (Estersen et al, 1969; Allison & Hahn, 1977), and to inhibit *in vitro* nucleic acid synthesis directed by DNA templates (O'Brien et al, 1966). Quinine was also found to inhibit DNA and RNA synthesis in *P. Gallinaceum* (Schellenberg & Coatney, 1961), and to be bactericidal for *B. megaterium*, although many species of bacteria appear to be unaffected by the drug, (WRAIR Annual Reports, 1969-1970). In *D. pneumoniae*, DNA, RNA, and protein synthesis were equally inhibited, suggesting that the inhibition of macromolecular synthesis was a secondary effect of quinine. The present mode of action studies on mefloquine were undertaken after a series of screening experiments showed that low concentrations of the drug inhibited the growth of many species of bacteria. Concentrations of mefloquine between 5×10^{-5} M and 1×10^{-4} M inhibited growth of *E. coli* B, *E. coli* K-12, *E. coli* AT-9, *Shigella flexneri*, *Staphylococcus aureus*, and Group D Streptococci. Growth of *Pseudomonas aeruginosa* was partially inhibited by concentrations of mefloquine between 5×10^{-5} M and 2×10^{-4} M, while the latter concentration was unable to prevent growth of *Klebsiella pneumoniae* and *Proteus vulgaris*. We have found mefloquine to be a potent bactericidal drug, and have obtained evidence suggesting that it may damage or alter a cell membrane. Exponentially growing cultures of *E. coli*, exposed to a concentration of 1×10^{-4} M mefloquine, immediately cease replication and uptake of isotopically labelled nucleic acid and protein precursors, and suffer a 34% loss in viability. Subsequent to drug addition further loss of viability occurs. Nucleic acids and proteins, or their constituents are released into culture medium and turbidity decreases continuously (Figure 1). Within four hours, bacteria lose 50% of their DNA, 40% of their RNA, and 25% of their protein. At a concentration of 3×10^{-4} M, mefloquine causes an immediate decrease of 99% in bacterial viability, with further decreases occurring in the subsequent four hour incubation period. Short term labelling experiments utilizing (^{14}C)-phenylalanine, (^{14}C)- uracil, and (^{3}H)-deoxyadenosine, showed that a concentration of 1.6×10^{-4} M mefloquine suppressed uptake of these macromolecular precursors within one minute after drug addition. The influence of the drug was very rapid, but again uniform suppression of precursor uptake was observed. Exposure of bacteria to concentrations of mefloquine between 3×10^{-5} M and 6 x 10-5 M also indicated that suppression of isotope uptake and inhibition of macromolecular synthesis occurred uniformly, and that mefloquine did not exert any specificity with respect to inhibition of macromolecular synthesis.

Our observations suggest that mefloquine possibly causes a rapid alteration in the intergrity of the bacterial membrane. THis hypothesis was tested through the use of N-tolyl-a-naphthylamine-8-sulfonic acid (TSN), a compound which fluoresces upon conjutation with protein. In a similar study this compound had previously

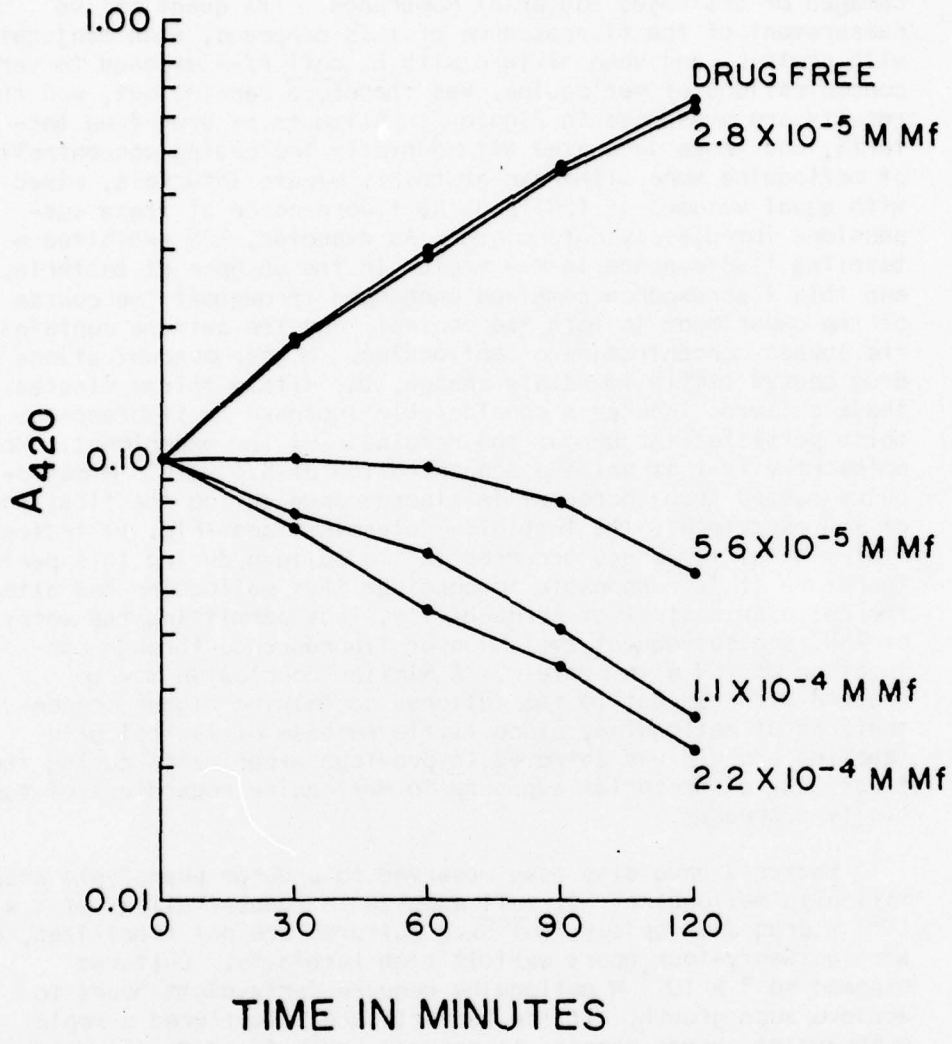


Fig. 1 The influence of graded concentrations of mefloquine upon the growth of E. coli AT-9. Growth and lysis of bacteria were followed by determination of the turbidity at 420 nm of aliquots of drug-free and drug containing subcultures. The specific, depicted observations were made in conjuction with the detection of permeability changes in bacteria induced by mefloquine and illustrated in Figure 2.

been employed by Newton (1954) to demonstrate that polymyxin damaged or destroyed bacterial membranes. The quantitative measurement of the fluorescence of this compound, when conjugated with protein, and upon mixture with E. coli AT-9 exposed to varying concentrations of mefloquine, was therefore carried out, and the results are presented in Figure 2. Aliquots of drug-free bacteria, and those incubated with serially increasing concentrations of mefloquine were withdrawn at thirty minute intervals, mixed with equal volumes of TSN, and the fluorescence of these suspensions immediately determined. As expected, TSN exhibited a baseline fluorescence in M-9 medium in the absence of bacteria, and this fluorescence remained unchanged throughout the course of the experiment in both the control, and the culture containing the lowest concentration of mefloquine. Higher concentrations of drug caused little immediate change, but within thirty minutes these cultures induced a considerable increase in fluorescence which persisted throughout the remainder of the experiment. Most noteworthy is that while a concentration of 5.6×10^{-5} M mefloquine caused such increases in fluorescence during the first hour of the experiment, the turbidity determinations (Fig. 1) indicated that minimal lysis and occurred in the culture during this period. Therefore it is reasonable to conclude that mefloquine had altered the cellular control of permeability, thus permitting the entry of TSN, and subsequent evolution of fluorescence through conjugation of TSN with protein. A similar conclusion may be reached with respect to the cultures containing higher concentrations of mefloquine, since little release of isotopically labelled protein was detected in previous experiments during the first hour of bacterial exposure to mefloquine regardless of turbidity decreases.

Bacteria have also been observed to undergo phenotypic adaptation to mefloquine. E. coli exposed to concentrations of 1×10^{-4} M drug die rapidly, but such cultures are not sterilized, and within twenty-four hours exhibit high turbidity. Cultures exposed to 3×10^{-4} M mefloquine require forty-eight hours to achieve such growth. If the bacteria are subcultured a rapid exponential growth appears to resume. When fresh drug is introduced into this culture, bacteria continue to replicate unabated. These bacteria now not only grow at a normal rate in the presence of mefloquine, but also in the presence of hitherto bactericidal concentrations of quinacrine (Ciak & Hahn, 1967), a potent antimalarial drug. The requirement for continuous drug pressure to evoke resistance in bacteria bears an interesting parallel to the induction of resistance among laboratory strains of plasmodia. The mode of action of mefloquine inferred from these studies remains to be rigorously tested in both bacteria and plasmodia, but investigations towards this objective are planned.

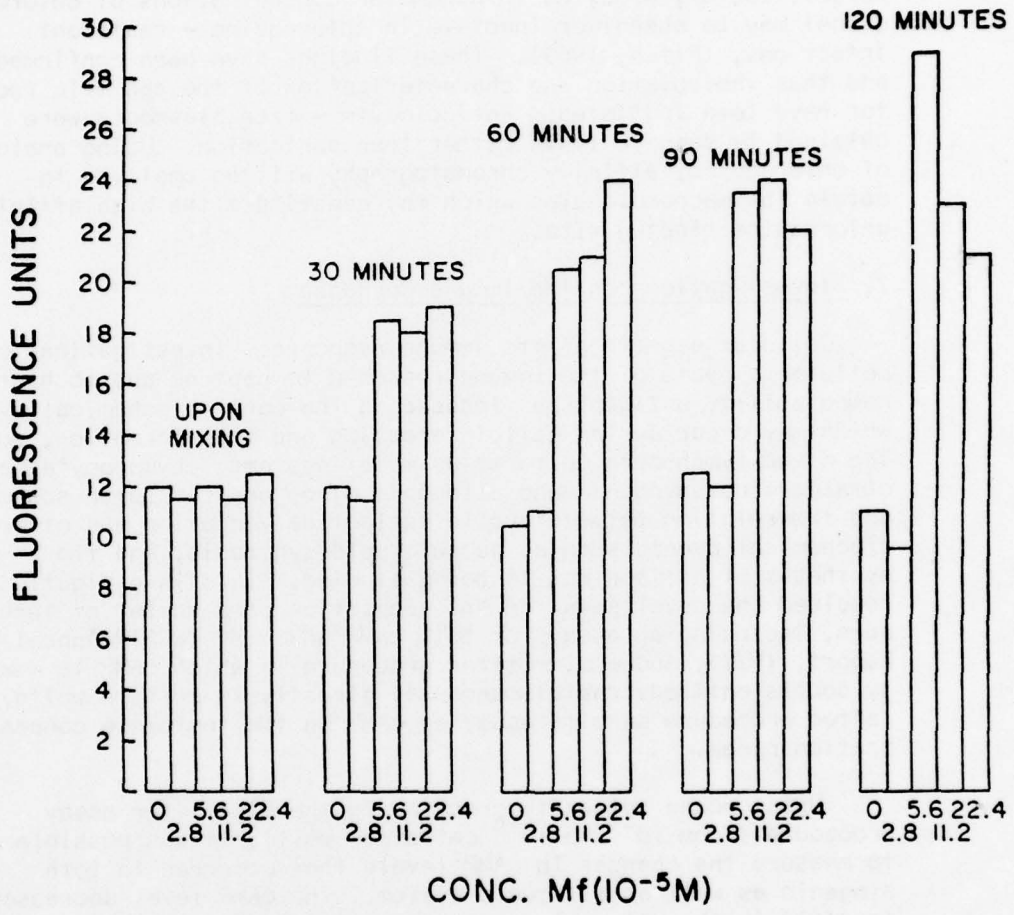


Fig. 2 The fluorescence of TNS upon mixture with standardized aliquots of subcultures containing graded concentrations of mefloquine, and in the absence of drug. Subculture aliquots were withdrawn at thirty minute intervals, their turbidity at 420 nm determined spectrophotometrically, and then standardized to the turbidity of the most dilute subculture. A volume of 1.5 ml of diluted bacteria were mixed with a volume of 1.5 ml of 4×10^{-6} M TNS dissolved in M-9 medium, and the fluorescence determined. The turbidities observed during the course of the experiment are plotted in Figure 1.

Studies on the possible mechanism of resistance of plasmodia to Chloroquine.

Parasitized erthrocytes posses three binding sites (Fitch, 1969) Whic differ in their affinity for chloroquine. The site having the greatest affinity (demonstrable only by exposure of parasitized erythrocytes to nanomolar concentrations of chloroquine) may be absent or inactive in chloroquine - resistant infections, (Fitch, 1969). These findings have been confirmed and thus theisolation and characterization of the specific receptor have been initiated. Reticulocyte - free plasmodia were obtained by saponin lysis rather than sonication. Using analoques of chloroquine, affinity chromatography will be employed to obtain the macromolecules which may constitute the high affinity chloroquine binding site.

2. Investigations on the Immune Response

Cellular aspects of the immune response. Investigations on the cellular aspects of the immune response to haptens and to histocompatability antigens has focused on the early biochemical events which may occur during cell interaction and transformation, employing mixed lymphocyte cultures as model systems. Lymphocytes are obtained from syngenic and allogenic mice, or from human sources, and the relation between cyclic nucleotide variation and other biochemical events such as nucleic acid synthesis, and the synthesis of antibodies, is being studied. This investigation has required the development or the adaptation of a number of techniques, including an assay for both cAMP and cGMP (WRAIR Annual Report, 1977), and a microtiter procedure in which cAMP is measured by double antibody radioimmunoassay directly in culture wells. This latter procedure permits assay of cAMP in the femtomole concentration range.

Using mouse spleen lymphocytes in the microtiter assay procedure (from 10^{-5} to 10^{-6} cells per well), it was possible to measure the changes in cAMP levels that occurred in both syngenic as well as allogenic system. The cAMP level decreases fourfold in the allogenic when compared to syngenic system. Similar changes in cAMP levels were observed in human mixed lymphocyte cultures. Several con stituents of the medium such as heparin and plasma may effect the level of cAMP changes for yet undetermined reasons.

Biosynthesis of Immunoglobulins. Many prerequisites exist to the development of cell-free systems capable of affording protection against militarily significant diseases. For example, sucessful in vitro synthesis of antibody may be accomplished only after the isolation and proliferation of a monoclonal cell line which produces high quantities of a specific antibody. In addition, advanced biochemical techniques must be adapted to these cells in

order to obtain functional in vitro systems, and thereafter to analyze their molecular products. Despite the complexity of this project, progress has been encouraging. Thus a hapten carrier has been defined, a cellular immune response to hapten has been demonstrated, a time course for humoral response to the hapten-carrier conjugate has been established by microculture, and the conditions determined for detection by plague assay of lympocytes that produce antigen specific antibodies. Additionally, antibody diversity has been demonstrated, and preparation of pure IgG and antibody fractions which retain full hapten-binding specificity has begun. When rabbit IgG containing hapten-specific antibody is applied to a polyacrylamide gel as free antibody, and protein separated by isoelectric focusing, a series of hapten-binding bands can be located by allowing the protein in 1 mm slices to elute and complex with (^{3}H)-hapten; the labelled complex can then be assayed by retention on nitrocellulose filters. The migration patterns of radioactive peaks indicate that the antibody species possess pK's which range from near neutrality to moderate acidity. When the IgG fraction was complexed with (^{3}H)-hapten prior to gel application, a different pattern of radioactive bands occurred from the extracted slices, with a general shift toward the free hapten at the cathode, and a possible change in intensity ratios. These changes, however, cannot be discerned in the seemingly identical staining patterns, Thus the heterogeneiety of our hapten-specific antibody can be demonstrated, and free antibody can be distinguished from the hapten-bound complex. Attempts to purify further and to fractionate the IgG by weak anionic exchanges have thus far resulted in the loss of hapten-complexing ability and/or loss of protein, however.

3. Prostatic Acid Phosphatase; Its Amino Acid Content and Employment in a Clinical Assay.

A clinical procedure intended to demonstrated the utility of radioimmunoassays (RIAs) in the detection of organ pathology has been developed (WRAIR Annual Reports 1975, 1976, 1977). Measurement by RIA of the modulation of the levels of the enzyme prostatic acid phosphatase (PAP) in serum and in bone marrow was employed to demonstrate this utility. Results have shown a significant relationship between increased levels of PAP and the occurrance of pathology or of the recent treatment of prostate glands. A high degree of significance was found between the increased bone marrow levels of PAP and the C, DI, and D2 stages of metastatic prostate carcinoma, while a lesser but still significant correlation was observed between the serum levels of the enzyme and these pathological states. Among the necessary proofs of the validity of the assay was the demonstration that the RIA measured the acid phosphatase which was synthesized by prostate gland cells. This objective was achieved when a correlation coefficient of

0.96 was obtained upon comparison of a portion of the amino acid content of PAP used to develop the RIA and of independently purified PAP (Derechin et al, 1971).

4. Determination of Drug and Metabolite Levels in Body Fluids and in Secretions, and the Potential Relation of their Modulation to Disease States.

The novel and/or improved methodolgy, developed for the purpose of biochemical investigations in the division of Biochemistry are tested and evaluated by providing and making them available (a) to other investigators at WRAIR on collobarative projects and (b) to various clinics at WRAMC for collaborative investigations.

Collaborative studies on antifolate activity of antimalarial and methotroxate determinations, (Hematology and Oncology clinic WRAMC and Div of Experimenial Therapeutics WRAIR).

A determination of the serum levels of the drug methotroxate in 124 patients suffering from leukemia and other malignancies was conducted within the past year. Approximately 3,000 individual assays have been performed. In addition to providing this clinical assistance, we have been able to apply the methotrexate assay to towards elucidation of the mechanism of action of a group of antimalarial drugs purported to inhibit the enzyme dihydrofolate reductase. A putative binding of these compounds to the enzymes has been tested through determination of the concentration of the experimental drug necessary to displace 50% of the methotrexate from its complex with dihydrofolate reductase from Lactobacillus casei. The following table presents results showing that seven of these compounds displace methotrexate from its enzyme complex, and therefore apparently bind to the enzyme, while two compounds are unable to achieve this displacement.

Compound	50% Displacement Concentration (pmoles)	
Methotrexate	3.0	3.0
WR180872	85.7	85.7
WR5949	1.38 x 103	1.38 x 103
WR5473	110 x 103	110.0 x 103

WR159412	70	70.0
WR2973	10×10^3	1.0×10^3
WR99210	38	38.0
WR38839	5.6×10^3	5.6×10^3
WR150012	ND	ND
WR178448	ND	ND

A logit plot of the interaction of these compounds with dihydrofolate reductase is characterized by a plateau at approximately a logit of 1.0 (79% displacement of methotrexate). This plateau potentially reflects a difference in the affinity of the compounds for a second methotrexate binding site. Only the compound WR 2948 failed to yield a logit plot of this type. Therefore, in general, it appears that these drugs may achieve their antimalarial action through inhibition of plasmodial dihydrofolate reductase.

Radioimmune assay modification for serum bleomycin, (Hematology and Oncology clinic WRAMC).

We have adapted and developed the RIA described by Broughton and Strong (1978) for the drug bleomycin. The method utilizes antisera produced in response to a human serum albumin-bleomycin conjugate. Water soluble carbodiimide was employed to mediate the conjugation of bleomycin to human albumin, and several different conjugation procedures were attempted before a suitable conjugate was obtained. The product was analyzed spectrophotometrically by plotting the ratios of the extinction coefficients at wavelengths of 292 nm and 305 nm for various theoretical stoichiometries of HSA-bloemycin ranging from 0:1 to 40:1. The following calculations are provided as examples:

Chromophore	$\Sigma\epsilon 292$	$\Sigma\epsilon 305$	$\frac{\Sigma\epsilon 292}{\Sigma\epsilon 305}$
HSA	1.82×10^4	3.82×10^3	———
Bleomycin	1.05×10^4	7.66×10^3	———
0:1	1.82×10^4	3.82×10^3	47.6
Bleomycin 1:1	2.87×10^4	1.05×10^4	2.74
40:1	43.82×10^4	30.64×10^4	1.41

For the experimentally prepared conjugate, a ratio of 1.72 was obtained, which corresponds to a hapten number of 5. (^{125}I)- bleomycin was prepared by iodination of histidine as described by Broughton and Strong (1978). Serum was prepared from animals injected at multiple sites on the back with 100 ug of bleomycin human serum albumin in Freund's complete adjuvant. Antibody was detected by applying reaction mixtures containing 100,000 dpm (^{125}I)- bleomycin and 10 µl of a phosphate buffer system to a 0.22µ nitrocellulose filter. Normal serum controls were included. It is appropriate to note that in the process of purifying the (^{125}I)-bleomycin, two major bleomycin peaks were eluted from the G-25 Sephadex column. The first major peak represented approximately 50% of the covalently attached label, and was substantially less cross reactive with normal serum than the second major fraction which corresponded to about 25% of the labelled column effluent. The balance of the reacted (^{125}I) was contained in six peaks.

	G-25 Sephadex Fraction #	
Serum #	35 (1st major peak)	42 (2nd major peak)
4	58 400	48 500
NRS	412	2 481
cpm Bleomycin	82 600	69 000

The RIA protocol described by Broughton and Strong utilizes charcoal dextran to remove non-complexed bleomycin. The logit plot of the competitive binding data was linear between logit +2.8 and logit -2.8, which represents the range of 7ng/ml to 2ug/ml bleomycin. Bo was 20,000 cpm and total counts of (^{125}I) bleomycin was 30,000 cpm.

Analysis of serum histamine by high performance liquid chromatography.

(Collaborative study with the Division of CD-1 WRAIR)

The feasibility of the analysis of serum histamine by fluorescent derivatization and high performance liquid chromatography (HPLC) has been established. The problem now is to increase the sensitivity of the method so that the measurement of histamine in 100 to 200 ml of serum can be precisely and accurately performed. The initial studies used a Waters µ Bondapak C-18 column and eluted the fluorescent derivation histamine with PIC and acetonitrite. Several methods of derivatization were tried. Derivatization with 2-methoxy-2,4-diphenyl-3 (^{2}H) fluoranone precolumn produced multiple peaks with histamine and several amino acids. Derivatization with O-phthaldehyde (OPT) at ph 9.0 (borate buffer) post column produced a single

peak with histamine but the recorder baseline was noisy and hence effective detection levels were only ng on column (Benson, J.R. et al. 1975; Roth, M. 1971). The procedure of Shore, P.A. et al. 1959 was tried next. The histamine sample was made alkaline with NaOH, OPT in methanol was added, the solution was incubated, and then acidified with HCL. The procedure was sensitive (1 ng on column), but produced multiple peaks. Attempts to quantitate histamine using these peaks failed because linearity and reproducibility could not be obtained. Derivatization with 1-N,N^{1}-diethyl-amino-naphthaline-5-sulfonyl chloride was used (Doyl, Z. et al. 1972; Bayor E. et al. 1976). This method produced a single, well defined peak for histamine, was linear and reproducible, but the detection levels were only 2 ng on column. The present method is a modification of the method of Shore, P.A. et al. 1959, derivatizing with OPT, and chromatographing using a C-8 column with PIC and acetonitrile. The sensitivity is good (50 pg on column) and the results are linear and reproducible.

Work is containing on ways of improving the extraction of histamine from serum and the derivatization of the extracted histamine. The present method of extraction based on the work of Shore, P.A. et al. 1959 requires about 2 hours. Over 200 sera and 800 controls have been analyzed to date to verify analytical procedures.

The study of polyamine levels in the urine from normal and prostatic cancer patients was continued. Differences were noted in the polyamine chromatographic profiles in these subjects.

A new high performance liquid chromatographic (HPLC) procedure was developed for the analysis of urinary polyamines. With this method, an analysis can be performed in 30 minutes compared 90 minutes using an ion-exchange procedure, and the precision and accuracy are better. Since this new method has been introduced 80 samples have been analyzed.

The urines analyzed contained polyamines and diamines. Quantitative differences were noted in putresine, spermidine, and spermine. At the same time two peaks corresponding to 1,2-diaminoethane and 1,3-diamino propane were observed in the urine from the patients with prostatic cancer. While 1,3-diamono propane does occur in normal urine, 1,2-diaminoethane does not. This fact was noted also using the ion-exchange procedure.

The results of these analyzes are presently being compiled and analyzed to determine the significance of these finding in cellular injury.

Other collaborative and service projects were performed: (1)

The analysis of 36 brain tissue samples for polyamine (Dr Meyorhoff, Division of Neuropsychiatry). (2) The analysis of amino acids in 25 specimens (Dr. Haynes, Seato Laboratory and Division of CD&I).

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DA OC 6444	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	124
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Research on Military Diseases

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002300 Biochemistry 010100 Microbiology

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FISCAL YEAR PRECEDING 78	8	386
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19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: BROWN, J.E., CPT. MSC

NAME: LYON, J.A., CPT. MSC

21. GENERAL USE

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(U) Toxin (U) Immunoglobulin (U) Vaccine (U) DNA

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 124 Biomedical Research on Military Diseases

Investigators:
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Associates: James E. Brown, CPT, MSC; PFC William J. Cahillane; SP5 Steven E. Englesen; George R. Fanning M.S. Seymour Garson, Ph.D; Mary K. Gentry B.S. Darrell E. Griffin ILT, MSC; John C.T. Olenick Ph.D Sara W. Rothman, Ph.D; SP4 Bimal Sinha, Frank Stokes; SP4 Otis Tate, SP5 Richard Travis Diana Whitaker; Alan D. Wolfe Ph.D

The objective of this work unit is the development of diagnostic procedures, and the studies on immunological and chemotherapeutic protection of military personnel through purification, characterization, identification of macro molecules related to disease processes. The following studies were conducted:

1. Characterization of Shigella dysenteriae toxin.
2. Characterization of Clostridial toxins.
3. DNA relatedness among Enterobacteriaceae.
4. Isolation and characterization of coat proteins of trypanosomes.

1. Characterization of the toxin from Shigella dysenteriae.

In collaboration with the investigators in Depts of Bacterial Diseases and virus Diseases, of Div of CD & I, this project have been focused on the delineation of the molecular characteristics and the role of a toxin associated with Shigella dysenteriae in pathogenicity of enteric diseases of bacterial etiology. The toxin is distinct from and in addition to the enterotoric, polymolecular endotoxin common to all gram negative entric bacteria. Previous investigations have resulted in obtaining highly enriched preparation of toxin which appears to be involved at least as one of its mode of action, a potent inhibitor of in vitro protein synthesis in pro - and eukaryoric systems. Also a highly sensitive, precise and reproducible microtiter assay to accurately measure cytotoxicity of toxin was developed. During the pass year the investigations have been entended to (a) improve the isolation and purification of toxin procedures (b) elucidation of the mechanism of inhibition of in vitro protein synthesis by the toxin (c)

determination of some of its physical properties and the lethal spectrum (d) the effect of the toxin on endogenus cyclic nucleotide levels of the target cells, and (e) localization of the locus of the toxin with the source bacterium.

a. Purification of Shiga toxin.

Considerable effort has been expended to modify the previously developed procedure to enable large scale purification of toxin and to optimize the recovery of toxin in its native form. In general, the following procedure is adapted at the present. It holds a good promise for obtaining reasonable amount of pure toxin for further studies on its characterization and its role in patholgenicity.

The cell paste is suspended in buffer and subjected to disruption in French pressure cell. The resultant preparation is next centrifuged at 50,000 RPM for 3.5 hrs in model L ultra-centrifuge (50•2 Ti rotor). The clear supernatant was aspirated and fractional ammonitum sulphate precipitation of protein was carried out. The fraction between 28 - 50% (room temp) contained most of the toxin. The 28-50% cut precipiltate were resuspended in 0.075 M NaCl in 0.02 M Tris•HCl at pH 7•8, dialyzed exhaustively in the same buffer and applied to previously equillibrated DEAE-Sephadex A-50 column. The toxin was eluted with 0•10 M NaCl in 0•02 Tris - HCl pH 7•8 buffer. The fraction containing toxin activity as determined by assaying for cytotoxicity using microtiter assay procedure were concentrated using diaflo filtration procedure. The concentrated toxin was applied to Sephacryl S-200 gel filtration column and the column was developed with buffer containing 0•10 NaCl in Tris•HCl pH 7•8. The appropriate fractions containing toxin activity were concentrated. Several batches of toxin preparation processed up to this step were pooled and dialyzed against 0•010 M phosphate pH 6•0 buffer. The preparation was applied, next, to carboxcymethyi cellulose column, and eluated with a linear gradient containing 0 - 0•01 M phosphate pH 6•0. The tube employed for collecting the fractions contained buffer solution at pH 8•0 so that the exposure of toxin to lower pH was minimized. The toxin enriched fractions were concentrated and subjected to a preparative isoelectric focusing, (pH 5-8). The toxin containing fractions were pooled, the ampliolytes removed and concentrated. This preparation was stored in aliquots at - 70%.

A typical results of purification by this procedure is summerized in Table I.

The post-isoelectric focusing fraction was approximately 50% homogeneous as estimated by densitometric scan of an isoelectric focusing gel.

Table I

Summary of Shiga toxin Purification

Fraction	Activity CD_{50}mg Protein	Total/mg Protein
1. Post Ultracentifugation	1.6×10^4	8200
2. 28-50% Ammonium Sulfate ppts.	2.0×10^4	5300
3. DEAE - Sephadex A-50 pooled Fraction	1.6×10^5	150
4. Sephacryl S-200 gel filtration pooled fraction	6.3×10^5	10
5. *CM-52 column	1.2×10^7	1.6
6. Post IEF	5.0×10^7	0.6

The specific activity of Shiga toxin appears to be $1\text{-}2 \times 10^8$/mg protein when judged by cytoxicity assay of this and other preparations. Therefore Shigella dysenteriae I contains approximately 2 mg toxin/kg wet weight of cells. Further analysis of the purified toxin (Preparation obtained by this method) by analytical isolectric focusing showed cytotoxin activity in 5-6 protein-staining bands. Most activity was localized in one band with an isoelectric point at pH of 6.9. Cytotoxin activity was detected from pH 6.3 - 6.9. Analytical electrophoresis again revealed cytoxin activity in four protein-staining bands, with a large proportion of the activity concentrated in one band. Preliminary estimates obtained by chromatography on Sephacryl S-200 and Biogel A 0.5m columns indicated the toxin possesses a molecular weight between 20,000-40,000 daltons, which is somewhat lower than previous estimates (Thompson et al, 1971 McIver et al, 1977). Incubation of the toxin with sodium metaperiodate, a compound which destroys vicinal diols, completely eliminates toxicity, thereby suggesting that Shiga toxin contains carbohydrate residues critical to its cytotoxicity.

Several other approaches were made to improve the yield of purified toxin preparations. One of the approaches was to modify the growth medium in order to maximize the production of toxin by bacterium. At the same time the growth conditions were altered in such a way that the toxin may "leak" out into medium thus facilitating the recovery. The variation tried were iron, salt, ph and nitrogen source concentration variations. Toxin yield was also studied in various strains of S. dysenteriae, including 60R, 3818 O, and in two "leaky" mutants selected (by Dr. Peter Demski) for cell surface defects which cause leakage of periplasmic proteins, including Shiga toxin. Thus far the strains have been found to grown equally well on two commercial media, Syncase, and N-Z amine A medium and to produce equal amounts of toxin in these media, while a third medium ST has been found to support growth poorly but to yield a higher quantity of toxin per cell. The composition of these media are as follows: (1) Syncase: contains Difco casamino acids, glucose, 0.2 ug iron/ml, and is buffered at pH 6.8 by 0.07 M phosphate. (2) N-Z amine A contains N-Z amine A casein hydrolyzate, and less than 0.1 ug iron/ml. (3) ST contains yeast extract, no added glucose, a pH of 8.6, and 0.6 ug iron/ml.

The results of this group of experiments indicated that optimal production of toxin could be obtained for strain 3818 O in Syncase medium at a pH between 6.8 to 7.2 with a phosphate concentration of 0.07 M. Concentrations of phosphate of 0.14 and 0.21 M served to sustian growth of bacteria for more than 24 hours, yielding a higher titer of cells, and of toxin, but the greater toxin yield was attributed to lysis of more bacteria rather than to an increased quantity of toxin per cell. These investigations of the influence of the growth media on toxin production are continuing in the hope of determing an even more perferable combination of nutrients and conditions which will increase the yield and specific activity of the toxin.

An attempt was made to obtain the toxin from the culture broth of a "leakage" mutant strain. Large (36 titer) culture of "leaky" mutant of strain PL-85, was grown overnight in Syncase broth, pH 7.2, 0.07 M PO_4. Toxin was prepared from broth by centrifuging out organisms and filtering supernatant fluids through 142-mm diameter, 0.45 mu pore sized Millipore filters. Broth was concentrated from 30 liters to 400 ml using hollow-fibre concentrators. The concentrate had a titer of CD_{50} 1.6×10^4/mg protein. The preparation was further purified by $(NH_4)_2SO_4$ precipitation and Sephadex S-200 chromatography. Polyacrylamide gel electrophoresis, however, showed that it had no fewer protein bands than toxin preparation at the same stage of purification but prepared by disruption of whole bacterial cells.

b. Inhibition of in vitro protein synthesis by Shiga Toxin.

Previously it was shown that Shiga toxin inhibits protein synthesis from both eukaryotic and prokaryotic sources (Steinberg, et al, 1976; Olenick et al, 1977). Continued investigation of these effects has centered primarily upon the elucidation of the mechanism by which Shiga toxin blocks poly-U primed synthesis of polyphenylalanine in a system derived from Escherichia coli. Serveral observations were of importance in the determination of the mechanism of inhibition of protein synthesis by Shiga toxin: (i) the toxin inhibited polyphenylalanine synthesis only upon preincubation with the extracted biological components of the polymerization systems, and (ii) the target component proved to be the ribosome. The effect of the toxin on the binding to ribosomes of the various macromolecular components of the polymerization system was therefore determined, and the toxin was found to inhibit the binding of poly-U to ribosomes. Results of a typical experiment are found in Figure 1, which depicts the inhibitory action of the toxin with respect to both the binding of (^{3}H)-poly-U and the synthesis of (^{14}C)-polyphenylalanine. The extent of inhibition of mRNA binding and of amino acid polymerization are comparable, and clearly demonstrate a a cause-effect relation between toxin action on the attachment of the mRNA and the function of the system. These results were verified by electrophoresis of ribosomes and poly-U in the absence and the presence of the toxin. Figure 2 shows typical electrophoretic patterns of ribosome incubation mixtures in an agarose-2.2% polyacrylamide gel stained with "stains all" and scanned for absorbance at 570 nm. The profile of the toxin-free system contains a prominent shoulder which migrated more slowly than the main body of the 70S ribosomes, while the toxin containing system lacks the shoulder almost completely. The shoulder is usually a population of monosomes which apparently was unable to form in the presence of the toxin, i.e. the toxin inhibited the binding of poly-U to the ribosomes. One can estimate that a ratio of approximately 5 toxin molecules to 1 70S ribosomal couple were necessary to produce 50% inhibition of the polymerization system, suggesting that the action of the toxin was not catalytic, and that the affinity of the toxin for its binding site was weak. Therefore to study the binding of the toxin to the ribosome, aliquots of toxin were iodinated using chloramine-T procedure. Although the original toxin preparation contained approximately 1.8 mg protein per ml, dilution of the toxin through iodination and subsequent chromatography on a Sephadex G-25 column resulted in a preparation which was useful for binding studies but was to dilute to produce inhibition of polyphenylalanine synthesis. The salient results obtained using (^{125}I)-toxin preparation were: (1) the toxin did bind to the ribosomes, and (2) factors influencing the binding of the toxin to the ribosomes could be determined. Analysis of the binding of iodinated toxin to the ribosomes was carried out by (^{125}I) coeluting with the ribosomes in the excluded volume after chromatography on a Sephadex G-100 column. Iodinated toxin chromatographed in the absence of ribosomes yielded little radioactivity

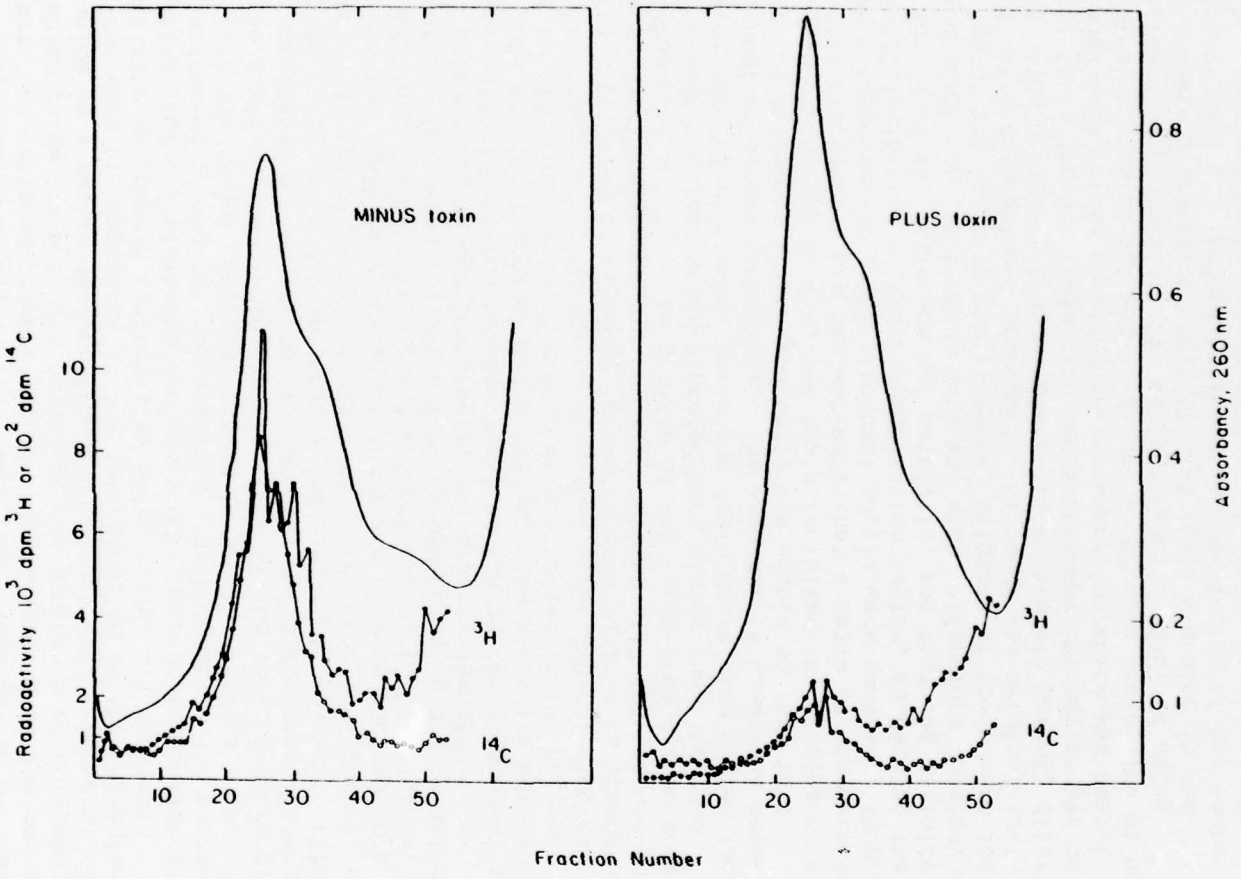


Figure 1. The simultaneous inhibition by Shiga toxin of the polymerization by E. coli ribosomes and elongation factors of (^{14}C)-phenylalanine and of the attachment to E. coli ribosomes of (^3H)-polyuridylic acid.

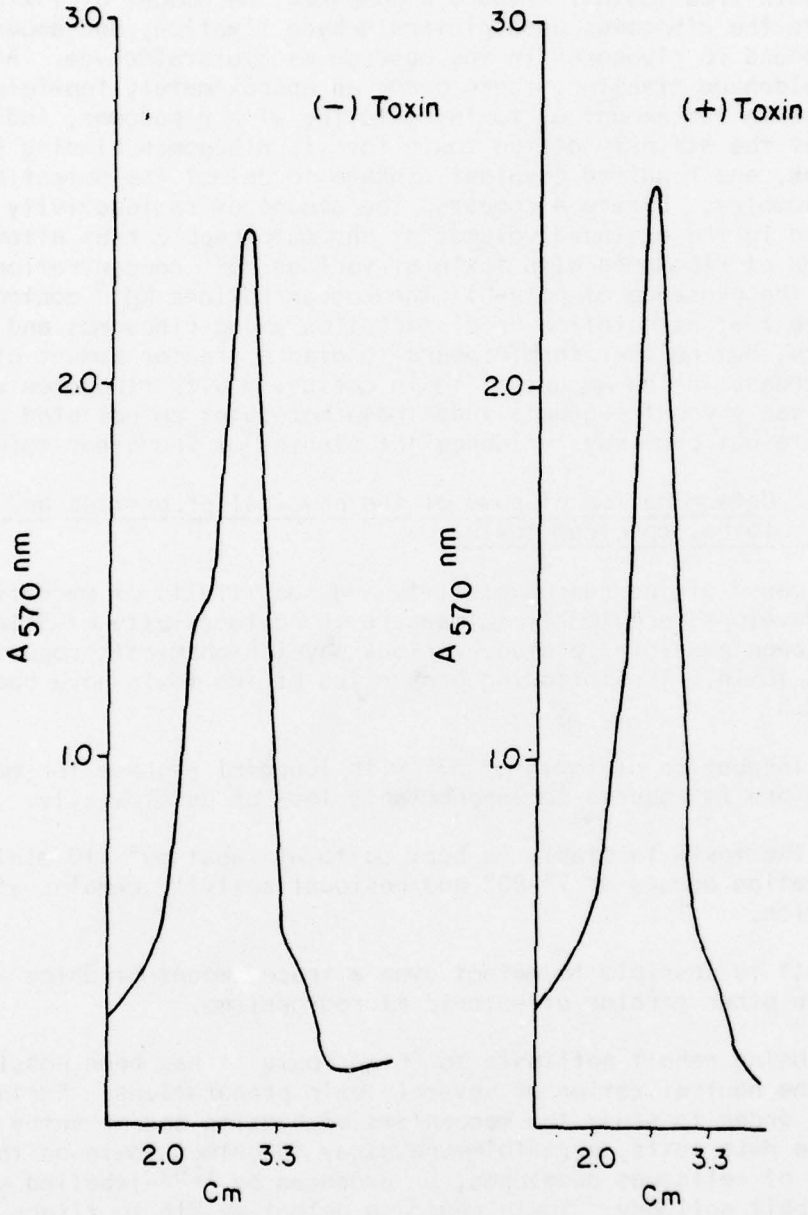


Figure 2. The influence of Shiga toxin on the agarose-polyacrylamide gel electrophoresis patterns of E. coli ribosomes and polyuridylic acid.

in the excluded volumn, but exhibited three radioactive peaks containing protein estimated to have molecular weights of approximately 50,000 and 100,000 daltons, respectively. A third peak appeared to contain free iodine. Figure 3 compares the amount of toxin bound to the ribosomes upon glutaraldehyde fixation, and amount of toxin bound to ribosomes in the absence of glutaraldehyde. After glutaraldehyde fixation, there occur an approximately ten-fold increase in the amount of toxin coeluting with ribosomes, indicating that the affinity of the toxin for its ribosomes binding site was weak, and required covalent linkage to detect the potential stoichiometry. Figure 4 compares the amount of radioactivity measured in the excluded volumes of chromatographic runs after incubation of ribosomes with toxin at various Mg^{2+} concentrations and in the presence of poly-U. The concentrations Mg^{2+} controls the extent of association or dissociation among ribosomes and their subunits, but neither form appears to bind a greater amount of toxin The increase in the amount of toxin coeluting with ribosomes when poly-U was present suggests that these molecules do not bind at the same site but each may influence the binding of the other molecule.

c. Determination of some of the physical properties and lethal spectrum toxin.

Because of the reproducibility and sensitivity of macrotiter assay developed previously to measure the cytotoxicity of Shiga toxin it has been possible to study various physico-chemical properties of this toxin. The following properties of the toxin have been elucidated.

(i) Incubation of toxin at 37° with 100µg/ml pronase for more than 18 hrs is requred for appreciable loss of cytotoxicity.

(ii) The toxin is stable to heat up to at least 69° (10 min). Partial inactivation occurs at 75-80° and residual activity remains after 90° incubation.

(iii) It is possible to detect even a trace amount of Shiga - like toxin in other strains of enteric microorganisms.

(IV) Using rabbit antitoxin to Shiga toxin it has been possible to study the neutralization of several toxin preparations. Further more in order to study the mechanisms of binding and/or entry of toxin into the HeLa cells, a radioimmune assay to detect toxin on the surface of cells was developed, or produced by ^{125}I-labelled goat anti-rabbit antibody. Toxin could be detect by RIA to titers of 10^{-4} - $10^{4.5}$, while cells exposed to toxin under the same conditions in the standard cytotoxicity assay indicated toxin at dilution of 10^{-6} - $10^{-6.5}$. The studies also indicate that toxin binding to HeLa cells is virtually complete within the first thirty minutes exposure.

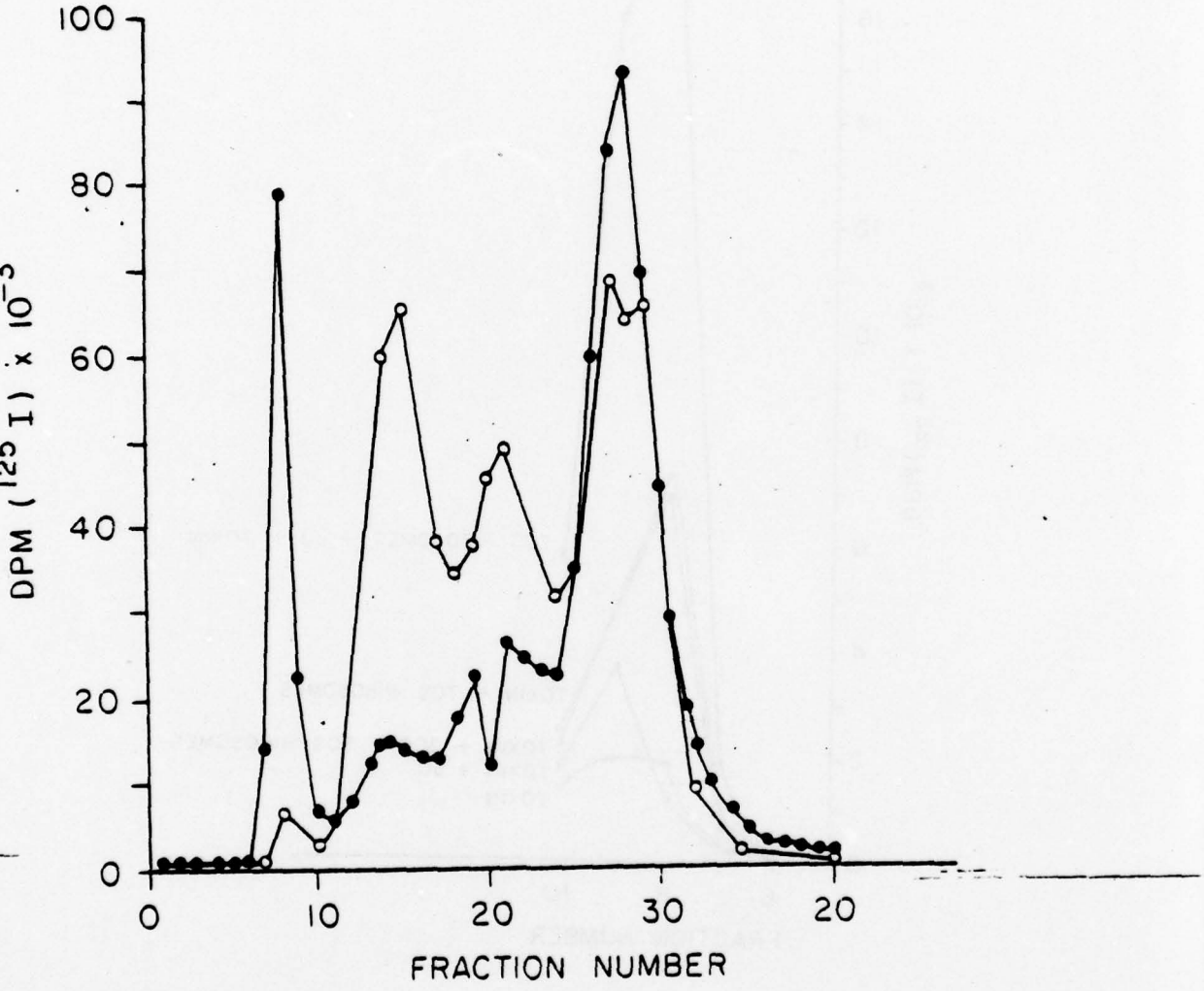


Figure 3. Sephadex G-100 fractionation of E. coli ribosomes and (^{125}I)-Shiga toxin after glutaraldehyde fixation. The ribosomes and co-eluting toxin appear in the excluded volume which consists of fractions six through ten. (·), glutaraldehyde present; (°), glutaraldehyde absent.

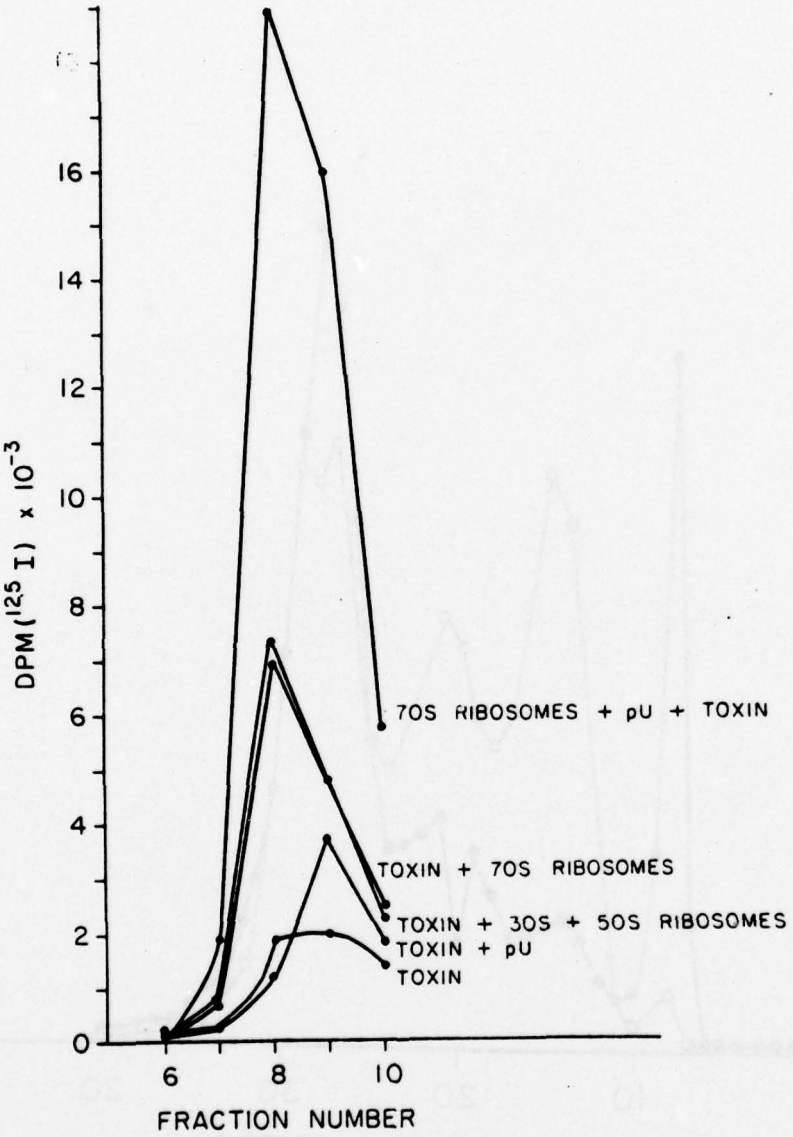


Figure 4. Sephadex G-100 fractionation of Shiga toxin in the absence and presence of associated and dissociated E. coli ribosomes and polyuridylic acid. The diagram depicts the fractions containing the excluded volume of the column.

A similar experiment using 60R strain toxiod indicate that the toxiod did not bind to the cell. The sensitivity of various human cell lines to the cytotoxic effects of Shiga toxin has also been studied. The effect of toxin on three different HeLa strains (the parent strain, strain S-3, and the Ohio strain), as well as the following cell lines, HEp-2 (human laryngeal epidermal carcinoma, FRhl, (monkey fetal lung), LLC-MK_2 (monkey kidney), H-VERO (monkey kidney), BHK (hamster kidney), L (mouse connective tissue), 407 (human intestinal), MA-331 (human intestinal), and KB (human oral epidermoid carcinoma) have been determined. Only the BHK line appears to be totally refratile, and no other line studied approaches the sensitivity of the HeLa cell.

d. Effect of Shiga toxin on endogenus cAMP levels in Hega cells.

Some microbial toxins, such as cholera toxin and Escherichia coli enterotoxin cause a cellular rise in cyclic AMP levels. A group has reported that Shiga toxin activates adenylate cyclase and increases cyclic AMP levels in ligated ileal loops of rabbits (Charney et al, 1976). Since cAMP synthesis is a cellular function, it is of interest to study the modulation of cAMP levels in HeLa cells exposec to Shiga toxin.

Tissue culture dishes, 60 x 15 mm, were seeded with 2.1×10^6 cells in a 3 ml volume. After allowing cell attachment overnight, highly purified Shiga toxin in a 0.33 ml volume was added and cells were incubated in the presence of toxin for various time periods from 15 min to 4 hr. Cyclic AMP was extracted from the cells with cold perchloric acid and levels determined with a radioimmunoassay. It was observe that in the presence of toxin endogenous levels of cAMP rose from a baseline average of 9.8 picomoles/mg cell protein to a high of 34.5 picomoles/mg cell protein in 2 hrs and then dropped precipitously to 5.2 picomoles/mg at 3 hrs and 1.7 picomoles/mg at 4 hrs. the lag before the rise cAMP levels suggests that the toxin must be internalized and/or modified before actin. The rise may be caused by activation of adenylate cyclase or by inhibition of phosphodiesterase. Attempts are underway to identify the cause mechanism of modulation of the levels of cAMP by Shiga toxin.

e. Release of Shiga Toxin without Cell Disruption; the Locus of the Toxin.

Presently the initial step in the purification of Shiga toxin involves French pressure cell lysis of the source bacteria. The resultant extract contains a large number of proteins inharent to bacteria, which complicate the problem of purification of the toxin. As a consequence, alternative methods have been sought to obtain Shiga toxin from cells, particularly procedures which cause release of the toxin without lysis of the bacteria. Two general methods

have been employed: 1) manipulation of intact cells, and 2) induction of mutant strains which release toxin into the growth medium. In addition, it was hoped that release of the toxin with the minimal cell disruption would identify the cellular locus of of the toxin.

Three different methods were utilized for the release of toxin from intact cells, these were: (i) treatment with polymyxin B, (ii) formation of spheroplasts, and (iii) osmotic shock. Treatment of bacteria with polymyxin was the most extensively studies of these methods because polymyxin has been shown to selectively release proteins and heat labile toxins from the periplasmic space of E. coli cell wall complexes (Cerny & Tauber, 1971, Evans et al, 1974).

Treatment of bacteria with polymyxin was found to release Shiga toxin from Shigella dysenteriae 3818 O. Release of toxin was achieved by modification of the procedures of Cerny and Tauber (1971) and Evans (1974), and involved the following steps: Overnight cultures of bacteria were diluted 1:10 into modified Syncase broth, incubated with shaking at 37 C for 6 hrs, and centrifuged at 10,000 xg for 15 min. The pellets were washed twice with 0.14M. NaCl, pH 7.3. The bacteria was then resuspended in 0.14M NaCl, pH 7.3 containing 2 mg/ml polymyxin B sulfate. After incubation for 2 and 60 min aliquots were removed and centrifuged at 16,000 xg for 20 min. The supernatants were assayed for marker enzymes and cytotoxicity in HeLa cells.

The cellular locus of Shiga toxin was determined by monitoring the release of toxin as a function of the release of cytoplasmic and protoplasmic marker enzymes. The enzymes 5' nucleotidase was used as a marker for proteins located in periplasmic space, while inorganic pyrophosphatase was assayed as the cytoplasmic marker. Approximately 50% of the toxin was released from exponentially growing cells after polymyxin treatment. It is not clear at the present whether the toxin is located in periplasmic space or not.

2. Characterization of Clostridial Toxins

Clostridial toxins have recently been implicated in the pathophysiology of antibiotic-associated colitis in animals (Bartlett et al, 1977) and man Larson et al, 1977). We reported previously (Katz et al, 1978) that clindamycin colitis in rabbits was accompanied by increased numbers of clostridia in the stools. Furthermore, sterile filtrates of stools from rabbits with clindamycin colitis caused severe epithelial necrosis and inflammation when injected into ileal loops or normal rabbits. These obaservations suggested that a bacterial toxin, probably of clostridial origin, was involved in the pathogenesis of clindamycin colitis in rabbits. In the studies re-

reported here, we demonstrate that this toxin in lethal to mice, cytotoxic to HeLa cells in culture and causes severe necrosis of rabbit rectal epithelium in vitro. The toxin can be assayed in the HeLa cell assay developed in this laboratory for studying Shiga toxin (Gentry et al, 1978). In addition, we have demonstrated that this assay is useful in the detection of Clostridial toxin in stools of humans with antibiotic-associated colitis.

a. Toxin from clindamyxin treated rabbits

(i) Lethal, cell-free filtrates of diarrheal stook from rabbit with clindamycin colitis caused death in mice following intraperitoneal injections. One-half ml volumes were lethal in dilution of 1:5. We previously showed that rabbits co-treated with clindamycin and vancomycin did not develop clindamycin colitis. Mice injected with stool filtrate from clindamycin-bancomycin in treated rabbits remained healthy, as did mice injected with 100mg clindamycin.

(ii) Cytotoxicity for HeLa cells.

Toxicity of filtrates was tested in a HeLa cell assay (Gentry et al, 1978), with serial dilutions of toxin added to Hela cell monolayers in microtiter wells. By 4 hrs after addition of toxin, cells were rounded; after overnight incubation, cells were detached from the monolayer. Activity could be detected through the 1:8 dilution.

(iii) Necrosis of rabbit rectal epithelium

The toxic effect of filtrates on rabbit colonic mucosa was measured in vitro in 18-hr explants. Addition of 0.05 ml of stool filtrates from a rabbit with clindamycin colitis to the organ culture medium resulted in severe epithelial cell necrosis and loss of architectural detail, while control explants were normal in appearance.

(IV) Properties of toxin

The toxin is a protein. Heating the stool filtrate for 30 min at 60 C completely abolished all three toxic activities. Precubation of filtrates with pronase (but not trypsin) at 37°C for 6 hr resulted in complete loss of mouse lethality. The toxin is non-dialyzable. Stool filtrates were chromatographed on Sephadex G-100 and toxic activity came off in one of two major protein peaks. By comparison with elution volumes of rabbit muscle aldolase (M.W. 158,000), ovalbumin (M.W. 45,000), chymotrypsinogen (M.W. 25,000), ribosnuclease A (M.W. 13,000) and glucosamine (M.W. 180), the molecular weight of the toxin was estimated at approximately 45,000 daltons.

3. Neutralization of toxin by Clostridial antitoxin

Because we have observed an increase in Clostridial species in the cecal contents of clindamyxin-treated rabbits, we postulate that the toxin in the filtrates might be of clostridial origin. Toxin filtrates before and after Sephadex chromatography were therefore preincubated with Clostridium perfringens antitoxins, types A through E, and with polyvalent gas gangrene antiserum. Clostridium perfringens E antitoxin completely neutralized the mouse lethal activity of the toxin; type A antitoxin partially neutralized. No other antitoxin had any effect, nor did normal horse serum. HeLa cell and rabbit mucosal toxicity could also be neurtalized by Clostridium perfringens E antitoxin; cytotoxicity was not neutralized by Cl. perfringens A antitoxin. Because Clostridium sordellii antitoxin neutralized the toxic activity in hamsters and humans with antibiotic-associated colitis, we tried neutralizing the filtrates with this antiserum. HeLa cell cytotoxicity was not neutralized by Cl. sordellii antitoxin. Broth cultures of Cl. perfringens type E, ATCC 27324 were toxic to HeLa cells. The data suggest that the toxin in the rabbit model of clindamycin colitis is produced by Clostridium perfringens type E.

a. Toxin from human antibiotic-associated colitis

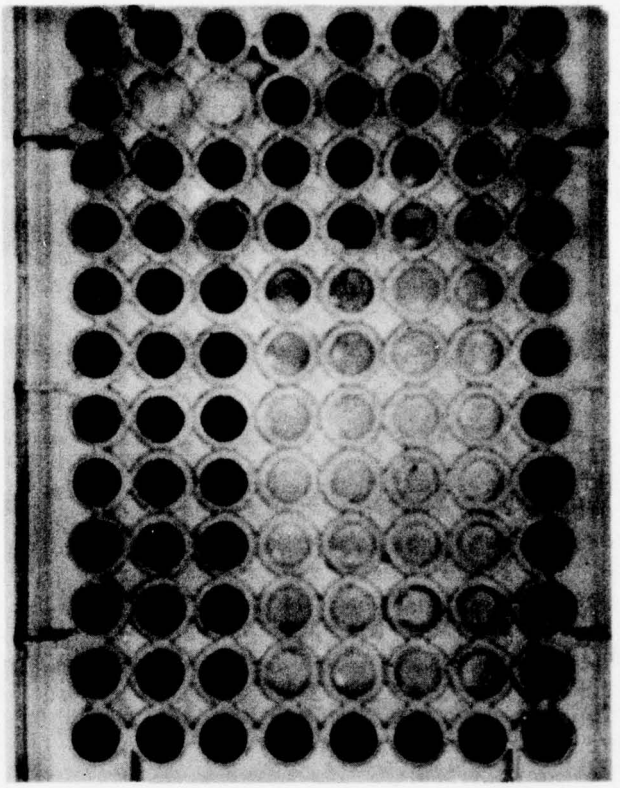
In the patients receiving antibiotics there is the hazard of antibiotic-induced diarrhea. With clindamycin, as many as 20% of the patients have diarrhea, and the incidence of pseudomembranous colitis has been estimated at 1-10% (Check, 1978). A clostridial toxin has been found in stool of patients with antibiotic colitis (Larson et al, 1977). This toxin is neutralized by antitoxin to Clostridium sordelliii. Anaerobic culture of the stools of these patients has revealed the presence of Clostridium difficile, which produces a toxin neutralized by antiserum to Clostridium sordellii

(1) Isolation of Clostridial toxin from antibiotic colitis patient.

The patient was a 49 year old woman with renal disease, who was admitted to Peter Bent Brigham Hospital in Boston with infection of her urine and was started on ampicillin, 500 mg by mouth, 4 times daily. Seven weeks later she was found to have diffuse pseudomembranous colitis (PMC) and she died ten days after.

The two different stools specimens were taken from this patient, and sterile extracts (A1 and A2) were tested for toxicity in the HeLa cell assay. Fig 1 shows that the extracts caused cell detachment at dilution of up to 1:512 (A1) and 1:128. Control specimen, A3, was from a patient with spastic colon; this caused cell detachment only in the 1:2 dilution. The toxic activity in the PMC extracts could be neutralized by Cl. sordellii. It was not

neutralized by any other antitoxin tested: Cl. perfringens types A and E, and type E, and type A enterotoxin, Cl. oedematiens, Cl. histolyticus, Cl. tetanus, Cl botulinum types A, B, and E, Vibrion septique, or Shiga.



$\overline{A}$ $\overline{A_2}$ $\overline{A_3}$

Fig I

Specimens A, A2 and A3 were diluted by 2-fold serial dilutions in cell growth medium. One-tenth ml volumes of each dilution were added to 2 wells each of microtiter plate with HeLa cell monolayers. Remaining cells cere fixed and stained after overnight incubating at 37 C in a 5% CO_2 atmosphere. Wells appear light in color where cells have detached due to cytotoxicity of extracts.

(2) Characterization of toxin

A sample of this extract was chromatographed on G-100 Sephadex and eluted with phosphate-buffered saline. Elution was monitored for protein (A_{280}) and for toxin activity in the HeLa cell assay. The toxic activity was eluted with one of the three protein peaks. Molecular weight is estimated at between 50,000 and 75,000 daltons. Cytotoxicity is inactivated by heating at 60 C for 30 min.

The effect of some microbial toxins is mediated by cAMP. We incubated the toxin with the HeLa cells for various time intervals from 15 min to 4 hr. Endogenous cAMP levels were measured using a radioimmunoassay (Schward-Mann). There was no change in the cAMP levels of the cells at any of the time periods tested.

These studies were carried out in collaboration with Dr. J. Thomas LaMont of Department of Medicine, Harvard Medical School Boston, Mass.

(3) Nucleotide sequence relatedness among Enterobacteriaceae

The enteric family of organisms contains pathogens which cause a variety of diseases including dysentery, enteric fever, urinary tract infection, food poisoning, and diarrhea. The enteric family also includes phytopathogenic bacteria. Our research is designed to determine the DNA relatedness of all organism in this family of bacteria. The data obtained is used to (i) develop a molecular definition of a bacterial species, (ii) characterize lines of evolutionary divergence in pathogenic bacteria, (iii) identify atypical clincial isolates for purposes of treatment and epidemiology, (IV) develop a classification based on genotypic relatedness and (V) accurately classify newly described organisms.

Previous studies in our laboratory have shown that enteric bacteria are all genetically related. In most cases there is a core of about 20-25% relatedness among these organisms. The exceptions are in the genera Proteus and Providencia, which exhibit about 10-15% relatedness to members of all other general Enterobacteriaceae.

Most of our effort during the past year has been focused on organisms in the genus Yersinia, which recently has been placed in the family Enterobacteriaceae. Although this genus is well recognized, increasing numbers of clinical isolates have indicated a need for additional epidemiological, biochemical and genetic studies. In addition our research has involved little known organisms in the enteric family which have possible clinical significance.

All of these studies are in collobaration with Dr. Don J. Brenner, Chief, Enteric Section, Center for Disease Control, Atlanta, Georgia.

DNA Relatedness in Sucrose-negative and Rhamnose-positive Yersinia enterocolitica.

The three species currently recognized in the genus Yersinia are Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis. There seems to be agreement that Y. pestis and Y. pseudotuberculosis are phenotypically related, and that both of these show very little biochemical variability. Brenner has shown that Y. pseudotuberculosis strains from sero-groups I through 6 were 80% or more related; additionally two other groups, Ritter and Gerlogg, and Moore and Brubaker have shown that a single strain of Y. pestis was more than 80% related to Y. pseudotuberculosis.

Strains of Y. enterocolitica differ substantially in their biochemical reactions, particularly in tests for indole, xylose, esculin, salicin, rhamnose, and sucrose. Previous studies in our laboratory showed that Y. enterocolitica strains with different biochemical patterns for indole, xylose, esculin and salicin showed an average DNA relatedness of 90%.

Two of the reactions used to distinguish Y. enterocolitica and Y. pseudotuberculosis are rhamnose and sucrose. Most strains of Y. enterocolitica are sucrose-positive and rhamnose-negative. However, some rhamnose-positive and sucrose-negative strains are now included in Y. enterocolitica or referred to as Y. enterocolitica like organisms.

Two groups of Y. enterocolitica do not ferment sucrose. One sucrose-negative group is otherwise typical biochemical. The second sucrose-negative group is part of a larger group of strains isolated from hares. These hare strains are atypically trehalose negative. They mimic the pathogenicity of typical human strains but seem to be host adapted to hares. The hare strains were 80% or more related to one another and to typical strains. The sucrose-negative, trehalose-positive strains were generally 60-70% related to both typical (tre^+, suc^+) and hare strains (tre^-, suc^-). Two groups could be distinguished within the environmental strains. These groups were about 75% interrelated and seem to correlate with serotype. Our conclusion is that the hare strains belong in Y. enterocolitica, and that the environmental sucrose-negative strains border on species levels relatedness to Y. enterocolitica. They are also separable from typical Y. enterocolitica in source of isolation, lack of enteropathogenicity, and in entent of DNA relatedness, howver, at the present time they will be included in the species Y. enterocolitica. (See included chart).

Additional work was done with rhamnose-positive strains and following results obtained. Strains which are $rhamnose^+$, $raffinose^+$,

and melibiose$^+$ or raffinose$^+$ (considered to be a rhamnose$^-$ mutant) from one DNA hybridization group, which are pathogenic to man, although different from typical Y. enterocolitica. Bottone and Chester have proposed the name Y. intermedia for these strains. A second of strains are only atypically rhamnose-positive. In general these strains are isolated from non-pathogenic sources. Two DNA hybridization are present, but as yet cannot be separate biochemcially. The name Y. fredericksenii has been proposed for these strains (see Table 2).

Escherichia blattae, a gram negative enterobacterium, Genetic relatedness to Enterobacteriaceae.

DNA hybridization was used to determined DNA relatedness among three bacterial isolates made from cockroaches on Rapa Nui Island in the southeast Pacific Ocean. Also tested was DNA relatedness of the Rapa Nui isolated to a single cocroach isolate from England, Escherichia blattae, and to species of Enterbacteriaceae. Relatedness of the Rapa Nui isolates to one another and to E. blattae was found to be very high (85% or more). Thus, all of the cockroach isolates are members of the same species. Biochemically these isolates are intermediate between E. coli and Hafnia alvei. They are 40 to 45% related to species of Escherichia, Salmonella, Citrobacter, Klebsiella, and Enterobacter and 25% related to Hafnia alvei. The genetic and biochemcal data are consistent with the designation Escherichia blattae.

4. Biochemical Studies of Trypanosomes

Antigenic Variation of Salivarian Tryparosomes

Tryanosomiasis or sleeping sickness poses a potential threat to the United States Army in its mission to deploy and maintain combat-ready troops in any area of the world. Because of the possible need for non-immune military personnel to operate in military theaters where trypanosomiasis is endemic, a better understanding of this parasitic disease, particularly of the phenomenon of antigenic variation, is required in order to design rational chemotherapy and immunoprotection against African trypanosomiasis.

Attempts at immunotherapy and immunoprotection are frustrated by the capability of the salivarian trypanosomes to undergo antigenic variation, thereby evading the immune response of the infected host. In chronic infections. the tyypanosome population continually changes the antigenic character of its glycoprotein surface coat in reply to the successive production of specific antibodies by the infected host. The variable antigen in a single variant-specific glycoprotein which forms a monolayered surface coat structure that adheres to the sytoplasmic membrane. The amino acid composition of the glytoprotein varies markedly from variant to variant, thus conferring a unique immunological identify to each variant.

The results of studies presented here and future biochemical/ immunological investigations are geared toward obtaining information on the nature of the control mechanism (s) and the molecular basis of antigenic variability. The specific aims are 1) to isolate, purify and characterize surface coat glycoprotein from a cloned strain of Trypanosoma rhodesiense and from a series (serodeme) of cloned antigenic variants derived from the original cloned strain, 2) to isolate and separate those polyribosomes involved in the synthesis of coar protein from the total population of polyribosomes and to extract from them the specific messenger RNAs coding for the polypeptide backbone of the coat glycoprotein and 3) to use the extracted and purified messenger RNAs to study the synthesis and elaboration of the coat protein and the genetic control and mechanism(s) mediating its production.

Trypanosomes are regularly isolated and separated from blood of infected rats by passage through a column of anion exchange resin under conditions where host blood cells were retained and trypanosomes were passed through (Lanham, 1968; Lanham & Godfrey, 1970). The use of DEAE-Cellulose was replaced by DEAE-Sephacel, which is in beaded form, requires no precycling and permits greater rates of flow. The procedure was scaled upward to permit one-step processing of approximately 300 ml of parasitized blood drawn from 30 rats.

Surface coat glycoprotein, which is known to possess the variant-specific immunological properties, is routinely prepared and purified by two methods. One method permits the isolation of cell coat; the other yields large undegraded polyribosomes and a supernatant enzyme fraction as well as a cell coat glycoprotein. Both methods, in part, incorporatd modifications of procedures of Baltz & Pautrizel (1976) and of Cross (1975).

Surface coat glycoprotein was removed at 4 C by overnight gentle agitation of trypanosomes suspended in 0.12 M sodium phosphate buffer, pH 5.5, containing 1 per cent glucose or by disruption by 3 cycles of freezing and thawing of the parasities (suspended in a sucrose-buffer -salts mixture, pH 8.5, consisting of 0.25 M sucrose, 0.2 M Tris-HCl, 0.05 M KCl, 0.05 M magnesium acetate, 0.006 m 2-meracptoethanol and 100 units per ml of heparin) followed by DNase addition (20 ug per ml) to markedly lower the viscosity of the lysate. After centrifugation (4,000 g for 30 min for the gentle extraction; for the disrupted, 10,000 g for 10 min then 150,000 g for 2 h), the supernatants were desalted on a column of Sephadex G-25 equilibrated with 0.04 M sodium phosphate buffer, pH 8.0, containing 0.03 M NaCl prior to chromatography on a DEAE-Sephacel column equilibrated with the same buffer. The sharp peak of material passing unretarded throught the column was collected and dialyzed against 0.5 M NaCl overnight at 4 C. Small amounts of aggregated material were removed by centrifugation at 100,000 g for 60 min.

The soluble extract containing 10-20 mg of protein was applied to a 10 ml Con A-Sepharose column previously equilibrated with 0.5 M NaCl. Unadsorbed protein was washed off with 0.5 M NaCl and the glycoprotein was then eluted with 0.5 M NaCl containing 10 per cent alpha-methyl-D-mannoside and 10 per cent ethylene glycol. The glycoprotein fraction was extensively dislyzed against distilled water at 4 C, concentrated to 1 mg protein per ml using immersible molecular separators (Millipore Corporation) and stored at -80 C.

A combination of immunoelectrophoresis and Ouchterlony testing revealed a single arc of precipitation and a line of identity with a cell coat glycoprotein sample prepared from the same strain but isolated by high salt extraction (mono-specific anterserum and the homologous glycoprotein were kindly supplied by the Department of Immunology, DCD&I). SDS-polyacrylamide gel electrophoresis followed by staining to detect proteins showed a single band with an apparent split peak corresponding to a molecular weight of 65,000; staining by the periodic acid Schiff method demonstrated the glycosylated nature of the preparation. In all samples of glycoprotein analyzed by SDS-polyacrylamide gel electrophoresis, a split peak was found. Since sufficient suggestive evidence exists to lead to the speculation that the cell coat glycoprotein is a secretory or exported protein and since such proteins have been shown (Campbell & Blobel, 1976) to be synthesized as larger precursors proteins, it is possible that the slightly heavier component of the split peak is a precursor form of the finished surface coat glycoprotein. Precursors of exported proteins contain an amino-terminal extension or signal sequence of 20-25 highly hydrophobic amino acid residues. The signal peptide sequence is removed by proteolytic cleavage during or after excretion. The glycoprotein preparation is presently being examined by hydrophobic interaction chromatography to determine if it contains and to isolate a component with an added hydrophobic sequence.

The pellet of the 150,000 g centrifugation contained both free and membrane-bound polyribosomes and was suspended in a sucrose-buffer-salts mixture, pH 7.5, composed of 0.25 M sucrose, 0.05 M Tris-HCl, 0 05 M KCl, 0.005 M magnesium acetate, 0.006 M 2-mercaptoethanol and 5 units per ml of heparin. An aqueous 10 per cent solution of sodium deoxycholate was slowly added with constant stirring to the polyribosome suspension to a final concentration of 1 per cent. The deoxycholate-treated suspension was then layered on a discontinous sucrose gradient of 0.7 and 2.0 M sucrose containing the same concentrations of buffer and salts as the suspension medium. Centrifugation was carried out for 4 h at 100,000 g and 4 C. The final pellets designated total polyribosomes were rinsed several times with the suspension medium and stored as pellets at -80 C.

Polyribosomes preparations were analyzed on linear sucrose gradients (15 to 30 per cent) and were shown to contain a wide distri-

bution of large-sized particles. The preparations were highly active in promoting incorporation of amino acids in a cell-free protein-synthesizing system. Sucrose density gradient analysis of reaction mixtures demonstrated that radioactive-labeled amino acids were incorporated into ribosome-bound nascent proteins which were then terminated and released. Immunoprecipitation methods will be employed to examine the products of the in vitro polyribosome-enzyme system to determine if cell coat specific polypeptide was synthesized.

Antiserum to cell coat glycoprotein prepared from a cloned strain (CP3B4) of Trypanosoma rhodesiense was raised in male New Zealand rabbits by injecting 1 mg of glycoprotein emulsified with an equal volume of complete Freund's adjuvant into 20 subdermal sites along the animal's back. At weekly intervals for a period of four weeks, the animals were given subcutaneous booster injections of 0.5 mg glycoprotein emulsified with an equal volume of incomplete Freund's adjuvant. Antiserum was titrated in microtiter plates using passive hemagglutination tests described by Herbert (1973) with erythrocytes conjugated to glycoprotein by the toluene-2,4-diisocyanate/glutaraldehyde procedure developed by Mahan and Copeland, Jr. (1978). Using antiserum, it could be shown that with homologous glycoprotein a single precipitin band was obtained in agar immunodiffusion tests and there was no interaction with heterologous glycoprotein prepared from a variant obtained upon chronic infection of a rabbit. In addition, the antiserum agglutinated homologous but not heterologous trypanosomes. Passive hemagglutination was observed only with erythrocytes coupled to homologous glycoprotein. A radioimmunoassay will be developed for use as a tool in identifying variants and in systematically monitoring the sequential appearance of variants in infected rabbits.

Clones and stabilates of clones have been prepared from strain CP3B4 of Trypanosoma rhodesiense. A clone of strain CP3B4 was injected subcutaneously into a rabbit and trypanosomes appearing at later stages of the chronic infection and expected to be distinct antigenic variants were sampled and stabilated. One such stabilate shown to be antigencially distinct from the "parent" CP3B4 strain. A complete series of stabilates is presently being prepared by sampling an infected rabbit at sufficient intervals to maximize the probability of obtaining a serodeme of distinct antigenic variants.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6457	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISS'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62771A	3E162771A804	00	048
b. CONTRIBUTING	61102A	3M161102BS01	00	125
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11. TITLE (Precede with Security Classification Code)

(U) Military Stress: Circadian and Ultradian Factors

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology 013400 Psychology

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76 07	CONT	DA	C. In-House

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b. NUMBER:

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19. RESPONSIBLE DOD ORGANIZATION

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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Achievement of an understanding of the temporal organization of biological functions attendent upon sustained exposure to stressors in military environments. Information developed provides indicators of the magnitude and time-course of stressor induced behavioral and physiological disorders that are the precursors of the production of psychiatric and combat casualties.

24. (U) Electronic and bioproduct monitoring techniques are employed in the laboratory and in the field to obtain detailed behavioral, electrophysiological, and biochemical measures of functioning during sustained operations. A variety of time series analysis techniques are applied to these data to assess changes that precede and accompany stress responses.

25. (U) 77 10 - 78 09 Three areas of laboratory and field investigation have been undertaken. A group of civilian firefighters is being studied to assess the role of occupational life threat and shift work in the production of psychological and physiological stress responses. A study of long range troop deployment and potential countermeasures against dyschronism induced by time zone changes was planned and undertaken. A field deployable technique for monitoring sleep is under field and laboratory evaluation. Preliminary results of these efforts are not yet available. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 OCT 77 - 30 SEP 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 125 Military Stress: Circadian and Ultradian Factors

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Description

This work unit is directed at the understanding of changes in the temporal organization of biological functions attendent upon sustained exposure to stressors characteristic of military environments. The information developed provides indicators of the magnitude and time course of stressor-induced behavioral and physiological dysfunctions that are the precursors of psychiatric casualties. Work is conducted in both laboratory and field settings to assess the biological costs of sustained performance requirements, rapid translocation across time zones and altered work/rest cycles.

Effort is expended on the development of the technology base required to acquire and analyze the long biological and behavioral data sequences needed to assess baseline, exposure and recovery functions. Non-invasive sensors and recorders designed for field deployment must meet rigorous requirements related to size, weight, ruggedness and reliability. The work involves both the adaptation of measurements previously restricted to the laboratory and clinic, e.g., the electroencephalogram, for use in naturalistic settings, and the development of new measurements that simplify the problems attendent upon field deployment.

Progress

1. Circadian Variations in Human Rest-Activity and Task Performance

Anticipated scenarios of future armed conflicts include requirements for continuous and as well irregular periods of operations. Soldiers will be expected to perform both rote and complex tasks: (a) during the night when mental performance and physical alertness are decreased; (b) while adapting to shifted rest-activity cycles; (c) following cumulative sleep

deficits; and (d) during periods of continuous re-adaptation to new sleeping environments.

These conditions are known to produce electrographically and subjectively disrupted sleep, as well as disturbances in normal human circadian and ultradian performance rhythms. Their practical implications might best be explicated by studying soldiers under field conditions. However, the Fire Direction Center Study (FDC) (See WRAIR Annual Report, 1977) has demonstrated the need to develop in the laboratory more robust and interpretable measures of psychomotor response, cognitive performance, and affective state. Furthermore the complexity of the FDC study has shown the limitations of present technology for recording physiological data, such as EEG, on freely moving subjects in a quasi-field situation. Finally, the multivariate nature of the experiment limited interpretability of findings and the explication of physiological and behavioral interactions. Thus, the relationship of changes in performance to associated temporal variations in bodily state must proceed from well validated laboratory measures which may in turn be applied in field situations.

A protocol entitled "Circadian Variations in Human Rest-Activity and Task Performance" has been designed to provide a bridge between basic human psychophysiology and field-oriented research. The major goals of this study may be summarized as follows:

(a) To assess the reliability and sensitivity of the Actigraph device as an indicator of rest/activity patterns, including sleep states.

(b) To determine the sensitivity of a speed/accuracy trade-off (SAT) performance task to circadian variations in bodily state.

(c) To determine the circadian variations of select cardiovascular and cortical events and their relationship to the information processing requirements of the SAT task.

(d) To determine the pattern of cardiovascular changes accompanying EEG-defined sleep states.

Several pilot runs were completed in the summer of 1978, ranging up to 48 hours of continuous measures. These runs have served to "shake down" the procedures, techniques, and personnel involved in this multidisciplinary research project. The laboratory is in a status poised to proceed with data collection, pending return of key personnel committed to the long-range deployment, or "Jet Lag" study, described below.

2. The Health and Effectiveness of Troops Deployed Over Long Distances: Countermeasures to Reduce "Jet Lag"

Research has been initiated to fulfill the CINC USAREUR's request to assess the impact of deployment upon soldiers rapidly transported across time zones. In conjunction with a USARIEM effort to evaluate the effects of "jet lag" on physical work capacity, a team from WRAIR is examining the effectiveness of chronobiologic countermeasures designed to hasten physiological adaptation to the new time zone.

The initial experiment is currently in progress with troops undergoing a permanent change of station from Ft. Hood, Texas, to Carlstadt, West Germany (2nd Armored Division Forward). Baseline data have been collected in CONUS on approximately 180 male soldiers. For the majority of subjects living off-base, the measurements consist of oral temperatures taken at 4-hour intervals during normal duty hours and upon going to bed and rising for four consecutive days. However, thirty subjects live in barracks and formed a special subgroup available every four hours for measurements of performance as well as temperature. Performance was assessed by two behavioral tasks: (1) responding for 8 min. on a Wilkinson 4-choice Reaction Time Device (Wilkinson, 1971); and (2) adding, as rapidly as possible, pairs of fifty random digits arranged in a column. Subjective fatigue was measured by having the subject complete an adjective checklist at the same time the other measurements were being taken. Throughout each day the special group also filled out a log describing when they ate, drank, slept, urinated, and had bowel movements. All these data are being analyzed by chronobiologic statistical procedures to determine the subjects home-base circadian rhythms before the six-hour shift to the new time zone. Oral temperature was chosen as the major dependent variable because it is considered as the "marker" rhythm in chronobiological research and because it correlates well with circadian performance rhythms.

Countermeasures will be utilized on ninety subjects (including fifteen from the special groups) in one airplane, while the second ninety subjects will serve as an unrestricted control group in a second plane. The planned countermeasures attempt to maximally accelerate the rate of rhythm resynchronization with destination time by manipulating social cues, energy metabolism, and rest-activity cycles on the day of departure, throughout the flight, and during the first day in Europe. The success of these manipulations will be evaluated by comparing the rate of phase shifting exhibited by the two planeloads of subjects during the first six days after arrival in Germany. During this period oral temperatures will be taken on all 180

participants every four hours around the clock. Those in the special group will continue performing the same tasks as during the baseline period. A preliminary report will be made to the CINC USAREUR by the end of November 1978 while planning is underway for a follow-on study to be carried out during REFORGER 79.

3. Psychological and Physiological Factors in Exposure of Life Threat

Military stress is an expression that denotes a complex constellation of interacting physical and psychological demands that modulate the health and performance readiness of soldiers. In the peacetime Army, the constellation of demand is virtually coterminous with that of the civilian population. The notable and non-trivial exceptions are those stressors arising out of the particularities of military functional organization and social structure. In time of war, preexisting stressors are intensified and, for the combat arms particularly, life threat enters the constellation of stressors. The goal of the present pilot study is to examine the influence of life threat on the structure of behavior, social organization and psychophysiological function.

We define life threat as a set of environmental features that are inimical to the continuation of an individual's life and that are so perceived by the individual. Explosions, flying missiles and fragments, fire, toxic gases, altered structural integrity of the surround, high wind and water, the enemy, etc. are some examples of life threat. All are situations where the safest course of action for an individual would be to remove himself beyond range of threat stimuli at the earliest opportunity. Yet certain occupational groups like soldiers, firefighters, policemen, bomb disposal teams and rescue squads regularly and voluntarily expose themselves to life threat in the disciplined performance of duty. We seek to understand the adaptations to and the psychological and physiological costs of engaging in such activities.

The scientific investigation of life threat to humans cannot be undertaken in the laboratory for obvious ethical and moral reasons. We can and do study factors that are often components of life threatening situations such as heavy performance task load, fatigue, information uncertainty and altered rest/activity cycles. In the recently conducted joint WRAIR/USARIEM study of artillery fire direction center teams, these factors were combined in a battle simulation. In each case, what the investigators could and would do to research participants was tempered by an overriding concern for the participant's welfare. Such studies yield

essential information concerning many aspects of the soldier's response to the demand structure of his environment. However, there is much information they cannot supply.

Men in hazardous occupations commonly undergo extensive and intensive training under conditions that are carefully controlled approximations to the ultimate occupational reality. Army field maneuvers are an example of this. Such maneuvers provide fertile ground for testing and extending what has been learned in the laboratory. Conversely, field observations and measurements can provide useful leads for laboratory investigation. They also open the first realistic opportunity for investigating life threat. Such training exercises are characterized by a heightened probability of accident and mishap that may lead to inadvertent exposure to life threat. However, accident and mishap are, by definition, unpredictable. Therefore, their investigation is necessarily retrospective in nature.

The prospective study of life threat requires the longitudinal investigation of groups for whom life threat is an occupational reality. Investigations of this type are subject to ethical restrictions that differ from those of the laboratory. For example, the rate of exposure to, or the intensity of life threat should in no way be influenced by the presence or absence of investigators. The research participants must be motivated and controlled by occupational concerns that are independent of and transcend their expectations of what investigators may or may not want.

While investigators need free access to groups under study, that access should not interfere unduly with normal functioning. In particular, situations should be avoided where, in the face of life threat, the group under study must divert a portion of its resources to keep the investigator out of trouble. Investigators must either be fully qualified as participants or be able to conduct their observations and measurements from a safe vantage point.

These ethical considerations interact with a number of very practical issues to influence the selection of an occupation for study. Occupation members should be readily available to investigators for observation and measurement. Members must be willing to serve as volunteer participants. The wearing of physiological monitoring equipment must be practicable. Baseline data should be obtainable. Threat exposure should be documentable and of sufficient frequency to permit the collection of a reasonable data base. The social organization of the group should be analogous to that of the military.

When all of these factors are considered, firefighting is

the occupation of choice for preliminary observational studies of life threat. Firefighters are available in every county and political subdivision. Fire departments are paramilitary organizations governed by extensive regulations and disciplinary codes. As recruits, rookie firefighters in the better departments are subject to rigorous physical screening, extensive training and continuing on-the-job training. In Montgomery County, Maryland, the proposed area of study, women have recently been added to the ranks of firefighters.

Firefighting is inherently episodic in terms of the demands it makes on participants. Periods of low physical and psychological demand are punctuated with episodes requiring rapid mobilization, deployment and exposure to potentially high physical and psychological demand. At the time a first alarm is sounded, the information available to firefighters is sketchy, including little more than the location of the fire and a general description of the structure and its involvement. Depending on the nature of the alarm one or more companies make the initial response. The remaining companies go on alert status, ready to respond to further alarms from the fireground or to provide backup for areas whose equipment is already deployed.

The potential character of any given fireground in a modern urban/suburban area is diverse. Structures range from single family dwellings; shops, businesses and light industrial plants; to high rise apartments. Potential firegrounds also include accident sites involving cars, trucks, trains, and planes. The combustion characteristics of the fireground are as diverse as the panoply of materials employed in the modern industrial state. These materials burn, explode, are inherently toxic or produce toxic combustion products, and may be corrosive. The presence of fire victims or potential victims on the fireground is a priority concern.

Upon arrival on the fireground the officer in charge must assess these factors rapidly and develop his tactics accordingly. The evolution of a fire can be extremely rapid and minutes lost may dramatically increase the degree of structural involvement of the fire. One important early decision involves a determination of the need to sound a second alarm to obtain reinforcements. Concurrently with this information processing and decision making, the crew is deploying equipment and beginning the fire combat. Deployment of equipment begins a period of heavy physical demand that will continue throughout the period of fire suppression and extinguishment, overhaul, and cleanup. The firefighters' overcoat, overalls, boots, hat and breathing apparatus weigh an aggregate 50 pounds. Water-charged fire hose is heavy and difficult to maneuver. Heat buildup under the protective clothing is rapid.

While the principles, strategies and tactics of firefighting are well known to experienced crews, translation into practice is heavily dependent upon the evolution of the individual fire. A high premium is placed on the ability of adapt one's behavior quickly and appropriately to rapidly changing circumstances. Communication on the fireground may be extremely difficult; visibility is often limited and high noise levels often prevail. Pairs of firefighters, or by error or mischance, individual firefighters, may be isolated from the rest of the crew. In such instances, individual discipline, skill and initiative assume an important role in determining survival.

In this brief introduction we have sketched out some of the salient features of the firefighters' task and environment. The reader who is familiar with military combat will recognize the analogous nature of the two undertakings. It is no accident that the language of firefighting is the language of battle. These parallels are the source of our fundamental hypothesis, i.e., by studying firefighters, we can learn things about military stress that will not be manifest in our laboratory or field training studies. We have undertaken two intersecting lines of investigation to examine the validity of this hypothesis: a) a psychophysiological study of the patterns of cardiac and motor activity rhythms exhibited by firefighters; and b) a psychosocial study of the cognitive, social, cultural and linguistic factors that mediate their stress responses.

It is well accepted that the classical physiological stress response is mediated by psychological, social, cultural and linguistic factors. Individuals, in and of themselves and as representatives and members of groups, perceive, order and respond differentially to the spectrum of internal and external stimuli that are normatively accounted against the cultural set "stressful". Thus, within the taxonomic or "emic" ordering of relationships, equivalent stimuli or the same stimulus in differing sociocultural contents might be responded to in a variety of ways, e.g., "stressful", "exciting", "exhalting", "ordinary", etc.

It has been hypothecated that individuals and groups engaged in hazardous occupations, e.g., soldiers, firefighters, policemen, and medical personnel, develop mechanisms that modify their responses to normatively stressful stimuli. These mechanisms might include selective inattention to certain classes of environmental stimuli that would normally be expected to elicit stress responses. In a more complex manner, culturally defined threatening and stress evoking stimuli may be subject to patterns of transformation into less threatening or stress evoking metaphoric representations of reality.

The analysis of physiological response patterns to stress should be correlated with the cognitive and symbolic mechanisms which chain and mediate them. A reliance on normative stressor models is inadequate and incomplete. The present state of the art of analyzing folk taxonomic and cognitive (or emic) systems dictates the use of qualitative and descriptive studies. These studies will attempt to determine and define both the patterns of taxonomic sorting and some measure of the weight, value, and available responses to environmental stimuli that are used by individual firefighters and their groups.

The fundamental questions being asked are:

1. What situations are perceived as legitimately stress evoking, within the language and consensual occupational values of the group?

2. Are there semantic fields surrounding such situations that are commonly assented to and that impart legitimacy to a range of responses to a range of stimuli that are subsumed under one representative class?

3. To what extent are cultural, linguistic and behavioral norms in both perception and response enforced by the group?

4. How much variation that is predicated on the singular life history of an individual is allowed for differential perceptions of events and responses?

5. Are there consistent group norms for metaphoric transformation of events and responses? That is, are there group sanctioned explanations of stimuli and responses an individual can make to himself that make them less threatening than they would normatively appear to be?

6. Are there consistent norms for regularized inattention to events and stimuli of high threat value?

7. When reconstructing high intensity and presumptively threatening events like a major fire or critical rescue, how much consensus exists within the group both in terms of description of the event and of their own and others' responses?

8. What are the differential roles played in response to stress by the singular life histories of individuals, and the social bonds and common culture of the occupational group?

9. Are stress reponses that are not played out within the group discharged in alternative environments, among family, friends, etc.? Are stress responses that arise externally discharged within the work group? In either case, are such displacements regularized or idiosyncratic?

A local fire department has been selected for study and all necessary arrangements have been made. The principal investigators have begun the process of establishing themselves with the firefighters and their officers. Interviews and physiological data collection are underway. The pace of this study is driven by events as they occur in the fire department. Emergency medical assistance and rescue operations occur regularly. Fires are less frequent. It is estimated that six to eight months of effort will be required before a sufficient data base is established.

4. Rest/Activity Monitoring: The Actigraph

The Actigraph is a wrist-mounted motion detection and data storage device that is finding increasing application to rest/activity patterns in both laboratory and field settings. As mentioned elsewhere in this report, electrographic (EEG) determination of sleep/wake cycles in field settings presents formidable technical difficulties. Even if good quality recordings can be obtained, the costs of data reduction and analysis are high. Finally, the required apparatus and associated electrodes are unacceptable in the field environment. Sleep logs require a degree of participation on the part of respondees that is difficult to acquire and maintain, especially in cases where mission demands create effective distractions. Therefore, we have invested developmental effort in a "wear and forget" unit, the Actigraph, that will have minimal impact on the soldier, that will provide interpretable data with respect to rest/activity patterns, and that will survive the rigors of the field environment.

We have constructed fifty (50) Mark I Actigraphs following modified plans for a device developed by National Institutes of Health engineering personnel. Our modifications had the effects of increasing battery life and adapting the device from ward to field use. The Mark I Actigraph is capable of storing 256 data words collected periodically over a 63-hour unattended period.

These units have been deployed in an in-house laboratory study (described elsewhere in this report), in the study of fire-fighters (described in the last section), and a collaboration with a USAMRDC contractor who is studying napping behavior.

Concurrently with the construction and deployment of the Mark I Actigraph, the initial development of the Mark II unit has been completed. Near the end of the reporting period, an agreement was reached with the Harry Diamond Laboratory to produce a hybridized prototype unit by January 1979 and to complete a production run of fifty (50) units by May 1979. The Mark II takes advantage of advances in electronic technology to increase storage capacity to 1024 data words and to increase the range of movement counts that can be stored. These modifications will permit a week of unattended recording and increase the recording sensitivity of nocturnal movements. In addition, the Mark II will be hermitically sealed and ruggedized to withstand the rigors of field deployment.

5. Multiple Complex Demodulation

Work was completed this year on the development of a quantitative method for the analysis of non-stationary biological time series. Biological time series have a complex periodic structure, i.e., the raw data can be decomposed into a set of waveforms differing in frequency, amplitude and phase. Some of these waveforms represent biological rhythmicities that characterize the functioning of the organism. Other waveforms are related to the waveshape of the biological rhythms. A third set of waveform components are regarded as representing a random noise component whose source may be the organism itself and/or the measurement system employed to obtain the time series.

In our view, a biological time series must be viewed as a dynamic rather than a static process. It is hypothesized that the essential features of the time series will vary as an orderly function of changes in the state of the organism that are produced both endogenously and exogenously. The main requirements for the quantitative analysis of such non-stationary time series are that:

a. Biologically significant waveform components can be identified.

b. Changes in these components can be detected, quantified, and related, where appropriate, to environmental events.

c. Statements relating to the statistical significance of identified components can be made.

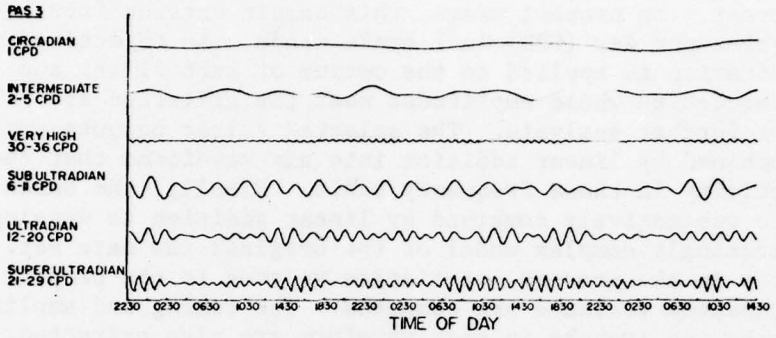
Multiple Complex Demodulation (MCD) meets these requirements. It is accomplished by synthesizing a series of narrow digital bandpass filters that span the frequency domain of interest. In present usage, this domain extends from 1 to 36 cycles per day (CPD) in 1 cycle steps. An objective amplitude criterion is applied to the output of each filter and those frequencies whose amplitudes meet the criterion are selected for further analysis. The selected filter outputs are then combined by linear addition into six waveforms that characterize activity in those frequency bands. Finally, the bands themselves are successively combined by linear addition to develop an increasingly complex model of the original raw data set. At each step in the process, statistics related to the proportion of accounted variance are computed. The timing and amplitude of peaks and troughs in each waveform are also extracted.

This process is illustrated in Figures 1a and b. The top line in Figure 1b, labelled Raw Data, is a 2.5 day Actigraph activity record from a single human subject. These data have not been smoothed or manipulated in any way. Periods of low activity related to sleep are readily identified. It is also apparent that activity occurs in a pulsatile fashion during the waking hours.

Figure 1a shows the waveforms of each of the six frequency bands that were discussed earlier. Except for the circadian (1CPD) and the ultradian (12-20 CPD) bands, the number of bands and their boundaries were arrived at pragmatically. The very high band (30-36 CPD) accounted for 3.8% of the total variance of the raw data. The subultradian band (6-11 CPD) accounted for 24.4% of the total variance. Within these extremes, the other four bands accounted for between 12% and 20% of the total variance. The three cirdadian minima, estimated by the MCD procedure occurred at 0400, 0345, and 0300. The period between the first and second minimum was 1425 minutes and between the second and third minimum 1395 minutes. These data indicate that this particular subject was undergoing a circadian phase shift during the period of observation.

The foregoing is presented as illustrative of the capacity of the MCD analysis to provide information about significant biological rhythmicities. Figure 1b deals with the issue of how well the MCD analysis models the raw data. The bottom waveform in Figure 1b is the sum of the bottom two waveforms in Figure 1a. The second waveform up in Figure 1b is the sum of the first three in Figure 1a, and so on. As Figure 1b is examined by scanning

a



b

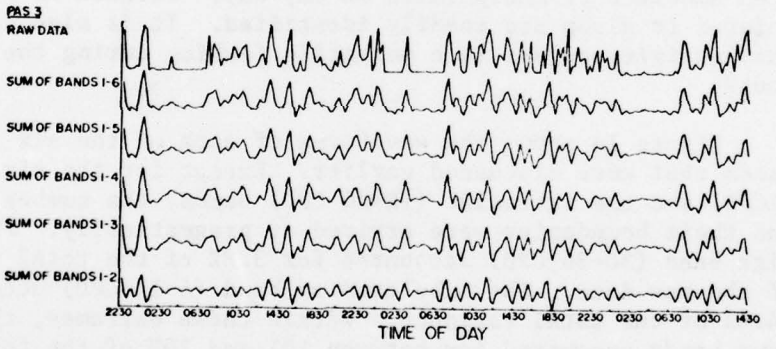


Figure 1a and b. Output of multiple Complex Demodulation analysis of activity time series from single human subject. 1a represents filtered waveforms in six bands convering 1 to 36 cycles per day. 1b represents the successive linear combination of the six band waveforms and the raw time series.

upwards from the bottom waveform it is apparent that the basic periodicities observable in the raw data are established in the first three summations. The mean period length in the ultradian band is 94.1 minutes (S.D. = 15.4 minutes), a value that agrees well with other reports in the literature.

At this writing, it appears that the contributions of the high, subultradian and superultradian bands are to the wave shape of the ultradian biological rhythms has not been extensively dealt with by investigators in the field of chronobiology. The most common theoretical assumption made is that the waveshape of a rhythm is sinusoidal. While this assumption may provide computational convenience, it may also blind us to important information. There are no a priori reasons why different chronobiologically organized systems need have the same waveshape or why the same system need have the same waveshape across time. MCD analysis provides a tool for investigating these questions and opens new avenues of approach to the role of biological rhythms in health and disease.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6451	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102B501	00	127
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biological Modulation of Military Performance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS 012900 Physiology 016200 Stress Physiology 013400 Psychology 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 06	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: N/A EXPIRATION:

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c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	7	455
FISCAL YEAR CURRENT 79	7	630

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Neuropsychiatry; (U) Physiology; (U) Performance; (U) Nerrophysiology; (U) Neuroanatomy; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Investigations will seek to describe the means by which the nervous system effects bodily responses to stress and injury, and to discern those combinations of physiologic parameters which collectively define the optimal conditions for effective military performance.

24. (U) Animal models of performance will be created using the techniques of operant and respondent conditioning and the role of internal factors in performance variability assessed by neurophysiologic recording of intracellular and extracellular bioelectric potentials; the descriptive and experimental neuroanatomical techniques of light and electron microscopy and histochemistry; stimulation or lesioning of discrete brain areas; and experimental modifications of hormonal status by ablation aud/or administration of exogenous hormones or other drugs.

25. (U) 77 10 - 78 09 Activities included: demonstration that subjects required to learn new tasks at night work at slower rates but without loss of accuracy compared to daytime. Demonstration that subjects in around-the-clock learning situations show rhythmicity in willingness to work, subject to externally imposed conditions. Description of the strategies devised by organisms to allow skilled movements following brain damage which permanently abolishes sensory input from the moving limb. Demonstration that cerebral cortex neurons involved in control of skilled movement and skin sensation may have reduced ability to function in face of noxious stimuli. Delineation of anatomic and physiologic characteristics of spinal cord neurons involved with cardiovascular function. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

* Available to contractors upon originator's approval

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 127 Biological modulation of military performance

Investigators:
Principal: Tyner, LTC C.F.
Associate: Hursh, CPT S.R., Manning, CPT F.J., Petras, J. M. Ph.D., Wylie, R.M. Ph.D., Elsmore, T.F. Ph.D., and Miller, M.G. Ph.D.

Description.

Work within this unit directs the technologies of multiple disciplines toward the analysis of biological modulation of military performance. The interaction of biology and behavior can be conceptualized on many levels. At one extreme is the manner in which the biological endowment of the normal individual controls behavioral adjustment to the military environment. This endowment may include how behavior is organized, how it is motivated, how it adapts to changes in environmental conditions, and how it may in fact alter the basic biological constituents of the individual. As a military medical research endeavor we are not only concerned with optimizing behavioral functioning but are also concerned with various so-called disorders of behavioral processes generated by a military environment; that is, why behavior becomes disorganized, unmotivated, or maladaptive. We tend to expect these disorders under conditions which appear stressful, noxious, complex, and demanding, conditions which for a variety of reasons are identified with military combat situations involving coping with life-threatening environmental circumstances, continuous or unusual scheduling of activities, and complex or conflicting task requirements. The first three specific activities described below address questions of how the normal biological endowment predisposes an organism to various forms of behavioral variations and breakdown when exposed to demanding or noxious environments.

At the other extreme are considerations of how alteration of the biological environment of the individual influences the normal structure and function of behavior. In this case we are concerned with the potential traumatic direct effects of a military environment on the nervous system and how that may in term disorder the behavior of the individual. For example, how the loss of normal limb afferents would alter motor functioning. Behavioral adjustment in this case is modulated directly by changes in the biological endowment and indirectly by the traumatic effects of a military situation. The last three activities described below address questions of how disruptions of the biological endowment may generate behavioral changes and what neural systems are important in mediating these disorders.

BIOLOGICAL RHYTHMS AND THE MODULATION OF BASIC BEHAVIORAL PROCESSES. The experiments covered in this section deal with orderly periodic fluctuations in behavior (i.e., behavioral rhythms). Recent technological

developments have increased the likelihood that military operations in conventional wars will involve essentially continuous round-the-clock operation, or operations at unusual times of the day. While it is well established that performance under such conditions will be subject to rhythmic fluctuations (circadian and ultradian rhythms), little is known of variables affecting such rhythmic processes, and little is known of dependent variables or behavioral processes most likely to show such variations.

What literature does exist on behavioral rhythms deals almost totally with behavioral output, or the rates at which behavior is emitted. The studies to be dealt with in this report are an attempt to investigate rhythms in aspects of behavior that are orthogonal to rate, such as the quality or accuracy of a performance. The following questions are of particular interest: 1) what types of behavior will show qualitative behavioral rhythms? 2) what variables are effective in modulating behavioral rhythms? and 3) what are the relationships (phase, amplitude, modifiability) between quantitative and qualitative aspects of behavioral rhythms? The question of the nature of the control of the rhythms, either by an endogenous biological clock or by exogenous entraining stimuli is not of particular concern to these studies, since it is assumed that the military operations to which the results of these studies may be applied would be carried on in environments containing powerful external entraining stimuli (e.g., day/night cycles). The fundamental strategy in all the experiments to be reported is to train animals to perform a task, then to test their ability to do so at various times of the day and night. All the experiments were conducted with primates, usually rhesus monkeys, as subjects.

Interoceptive stimulus control. When a behavioral response is more likely in the presence of one stimulus than in the presence of others, it is said that stimulus control has been established. The stimuli may be either exteroceptive, (e.g., a light or a sound) or interoceptive, (e.g., a visceral stimulus or a state of deprivation). It has been suggested that behavior controlled by interoceptive stimuli is more subject to disruption by a variety of pharmacological and toxic agents, as well as other environmental perturbations. Time-of-day effects on behavior might therefore be more profound when the behavior is under interoceptive stimulus control than when it is externally controlled. The present experiment established conditions for behavior to be interoceptively controlled. Four monkeys lived in experimental cages containing two keys. In order to get food, the monkeys completed at least 10 but not more than 15 presses on the first key (key A) before responding on the second key (key B). Switching to key B either too early or too late resulted in a 30 second period of time (timeout) in which food was unavailable. The stimulus for switching, therefore, was the completion of the required number of responses with no external cue provided. The behavior of switching therefore, must necessarily be under control of some interoceptive cue. To increase the likelihood that the internal cue was the number of responses completed rather than the amount of time spent responding, a required delay was imposed between successive responses on

key A. Each time the animal pressed key A, both keys turned red for a period ranging from 0.1 sec to 0.9 sec. Pressing either key during the delay resulted in a timeout. For any given run of key A responses, the delay remained the same.

The results of this experiment may be analyzed in several ways, the most promising of which is signal detection theory, which allows separation of the organism's sensitivity to the stimulus dimension controlling behavior from factors affecting the bias for alternative responses. Data thus far indicate that a circadian rhythm exists that is evident in some but not all aspects of their performance. First, there is an overall tendency to perform more quickly at certain times of the day, a quantitative rhythm. Second and more importantly, there is a clear rhythmicity in the production of errors (switching too soon) evident when the imposed delay between counting responses is long, the most difficult task; and the most errors occur when the subjects are most active! As a result, the percent correct with long delays was lowest during the active phase of the rhythm. This counter-intuitive result is more understandable within a signal detection account of the performance. First, most of the variance in performance resulted from shifts in the subjects' bias toward switching and not from shifts in the overall sensitivity of the subjects to the correct count; their "ability" to count seems stable. When the subjects were most active, they were also most anxious to press the switching key and receive their food. Although the peak of the switching distribution remained within the reinforced range of counts, the lower tail moved more into the non-reinforced range, increasing errors. Second, this effect was most evident when the imposed delay between counts was longest, the trials when overall errors were greatest and when the bias toward switching was strongest to begin with. This shift in bias toward switching was not a simple consequence of activation. For example, one subject had his shortest latencies at 0600 hours but had maximal shift in switching bias at 1400 hours. Also, inappropriate responding between trials was maximal at 0200 hours when the switching bias was lowest. Thus, while several aspects of performance were rhythmic, it is best described as a variation in the qualitative composition of performance rather than in the quantitative output of behavior.

Analysis of individual data indicated that the phase of the circadian rhythm, i.e, the time of day with greatest activity or maximal switching bias, was not the same for all subjects. One subject peaked at 1400 hours and two peaked at 2200 or 0200 hours. One subject showed a unique pattern of terminating trials with long inter-count delays, not by switching early, but by pressing the key during the delay. For him, the rhythmicity in performance was manifested in a shift in bias toward premature counting responses, rather than premature switching responses. These differences between individuals requires separate analysis of each subjects' behavior.

Microanalysis of fixed-ratio performance. Two monkeys were studied on simple schedules in which one food pellet was provided for every 50 key presses (FR 50). Sessions were conducted at various times of the day.

Extremely fined-grained data collection procedures were used in order to enable a detailed analysis of performance changes as a function of time of the day under these simple conditions. As in the previous more complicated counting task, not all aspects of this performance were rhythmic. The most obvious rhythm was found in the spacing of responses. During the most active times of the day, responses were more closely spaced early in each 50 response run. Spacing later in the run was much less rhythmic being rather closely timed at all hours of the day. Secondarily, it was found that the latency to start a run was also somewhat rhythmic.

Acquisition of new behavior. A third task has been developed which requires the subject to press three keys in a predetermined sequence of four responses. For example, food may follow a sequence of presses such as key 1, key 2, key 3, key 1. The correct sequence is changed at the start of each session so that the acquisition of the solution can be studied repeatedly. During the early part of each session the subjects typically attempt a variety of different solutions. Any correct response - such as key 1 in the last position of the example above - is signalled as correct regardless of the accuracy of the other three responses, but food only follows entirely correct sequences. The subjects achieve a high level of accuracy requiring twenty to forty attempts before solving the sequence problem consistently. A computer system of control and recording permits automatic programming of new problems without experimenter intervention and automatically records the details of each acquisition process. For this study, two sessions were conducted each day, with a new solution required in each session. The time of each session was randomly selected from six possible hours around-the-clock. When first placed on this procedure of rotating sessions, after consistent training at 1000 hours, response speed and solution accuracy was found to be more or less rhythmic in all four subjects, with peak efficiency occurring at 1000 hours. After three weeks of experience with this routine, the rhythm in responsiveness persisted but the rhythm in efficiency nearly vanished. Since the total daily ration of 150 pellets required accurate solution of both problems no matter what the hour, it was reasoned that providing a surplus of pellets would increase the likelihood of a rhythm in efficiency. Over the course of several months, the ration of food available in each session was increased in steps of 20% from 75 pellets to 156 pellets. The rhythm in responsiveness (speed) tended to increase but the accuracy of reaching solutions did not become more rhythmic. With 312 pellets available per day the subjects continued to eat nearly all the food. It was then reasoned that the random timing of sessions may be operating to reduce rhythmicity; the animals worked efficiently because they could not predict the time of their next meal. The 312 pellet daily ration was divided into 52 pellet portions available during six regularly scheduled sessions around-the-clock, each requiring the solution of a new problem. Now six predictable meals were available. The daily rhythm of activity was greatly increased, with sessions at 2200 or 0200 hours often passed over. When the animals performed, accuracy was no longer uniformly high. There is evidence of a correlation between low response rate and reduced accuracy, and by

implication, a rhythm in accuracy of solving problems. This condition is still being studied. The subjects are becoming obese on this rich schedule of food and will very shortly have to be shifted to a leaner diet. As the diet is reduced, the amplitude of behavioral rhythmicity should decline.

Delayed stimulus control. Complexity of behavioral procedures also increases their sensitivity to disruption by a variety of influences. Situations which impose a delay between a signal and the opportunity to emit one of two responses based on the nature of that signal are particularly difficult and sensitive to disruption. The four monkeys in this experiment are required to respond to terminate a train of tone pulses in which the tones are either shorter or longer than 0.6 sec. Following a delay period ranging from 1 to 9 sec, the animal has an opportunity to respond indicating whether the tones in the preceeding pulse train were long or short. Correct choices are followed by food, and incorrect choices, or responses during the delay period are followed by a timeout. Two of the four subjects in this study have completed a thorough evaluation with one session a day scheduled at one of six possible times-of-day. The data have not as yet been thoroughly analysed, but behavior with one session a day and only 100 pellets available appears to have no rhythm, either in inclination to work or accuracy.

The several studies described above thus show that certain critical aspects of problem-solving -- such as willingness to work, rate of task performance, and the tendency to commit errors -- can vary cyclically over time. They further show that variability is not necessarily present in all aspects of task performance, nor are the "high" and "low" periods identical for all individuals. These results might be viewed as supporting the idea that certain complex military tasks, possibly those involving soldier-machine interactions, must be performed in different ways -- depending on time of day, the individuals involved, and other factors -- to achieve optimum effectiveness.

THE BIOLOGICAL ECONOMY CONTROLLING CHOICE BETWEEN DIFFERENT COMMODITIES. Economic factors strongly influence the variability of behavior and the susceptibility of continuous performance to biological rhythm as illustrated by the results of our study of problem solving with various size rations and numbers of meals (above). Experimentation continues to explore the various fundamental economic principles - supply and demand, elasticity, substitutability, utility - with individual subjects in controlled environments. The current experiment will separate the effects of supply (or resource density) from the effects of price (or procurement cost). Technical difficulties as well as shifting emphasis have prevented this experiment from reaching completion during FY78.

SENSORIMOTOR CORTEX: ACTIVITY FOLLOWING NOXIOUS STIMULATION TO VISCERA AND SKIN. We continued during the past year to investigate an uncommon type of neuron which conveys the output of sensorimotor cerebral cortex to lower parts of the nervous system; these cells have traditionally been viewed as playing a crucial role in rapid, precise, "voluntary"

movements, particularly those of the hands. We have now been able to show that these cells are also selectively sensitive to noxious stimuli delivered to the viscera and also receive input from a spinal cord pathway presumed to have a special involvement with painful stimuli delivered to the skin. These neurons may be some of the CNS loci at which "stressful" events act to disrupt skilled motor coordination, and we are presently examining the pharmacologic modification of their behavior.

Additional work has involved the application of several multivariate statistical techniques to the analysis of data on the behavior of single brain cells; this has been done as part of a program to simplify and reduce greatly the volume of data which must be collected and examined in order to specify how a part of the brain responds to a given stimulus.

RECOVERY OF MOTOR FUNCTION AFTER LIMB DEAFFERENTATION (DORSAL RHIZOTOMY).

Surgical deafferentation of a limb by dorsal rhizotomy abolishes spontaneous movement but by appropriate experimental intervention, movement can be restored. We are comparing the performance of normal and unilaterally deafferented monkeys on a task in which the animals must compensate for an external load as they flex their forearms through a criterion arc in order to achieve reinforcement.

One deafferented monkey reached the end of his experimental life and was sacrificed. Histological sections of his spinal cord have been prepared and will be reviewed to verify the completeness of the rhizotomy. A second deafferented animal is currently undergoing testing. Results from this animal have largely confirmed results obtained from the first animal.

Both animals were initially trained with no external load and then challenged for the first time with a load that was in the middle of the working range previously established for normal animals on this task. Whereas the first deafferented animal we tested reached criterion on his second lift, the second animal required six attempts before he was able to move his arm off the rest position and obtained a reinforcement only after 59 responses. That both animals were initially impaired supports the intuitive assumption that the test load in fact required a change in the neural signals controlling the muscles.

Both animals, when tested over a range of loads make the long latency corrections in the trajecting of the arm that has proved to be a distinctive difference between the normal and deafferented animals. On the first lift of an unknown load both normal and deafferented animals achieve about the same regulation of final position, but the deafferented animals achieve this only by a delayed correction. The inference is that normal animals to some extent utilize servo loops in the execution of the task.

Muscle fatigue has proved to impair the performance of the task by deafferented animals. Normal animals do not generate errors attributable to muscle fatigue but interrupt their responding at intervals. Both deafferented animals tend to continue responding until the local density of reinforcement drops to zero. The conditional probability that a non-criterion response will be followed by an increment falls from the start to the end of a run of responding. The conditional probability that a non-criterion response will be followed by a criterion response falls still more steeply at the end of a run. When programmed and signalled timeouts are provided and when response rates are externally paced, the performance of deafferented animals is more nearly stationary through a session and terminating sequences of unreinforced response occur less frequently. These observations provide a strong argument that signals related to the state of muscle fatigue contribute, in normal animals, to the avoidance of fatigue states that would impair performance.

STUDIES OF AUTONOMIC PATHWAYS.

We continue to pursue studies of the anatomic pathways of the autonomic nervous system as a base of technical data for concurrent studies of psychosomatic disease, autonomic function and evaluation, and recovery of function after injury. Significant progress has been made in perfecting neuron staining techniques for peripheral and spinal pathways. As a result the following findings can be reported:

1. Output from the spinal preganglionic neurons of the sympathetic and parasympathetic autonomic nervous system seem to show viscerotopic organization. For example, parasympathetic preganglionic neurons innervating the bladder originate primarily from the ILSp and ILSf nuclei, whereas the urethra is richly innervated by ICd neurons.

2. Using horseradish peroxidase injections of intact and isolated paravertebral ganglia, morphologic and architectonic description of preganglionic sympathetic neurons were made. A previously undescribed intraspinal sympathetic preganglionic pathway (ISPP) was found. Follow-up physiologic studies revealed the precise localization of this pathway within the spinal cord and showed that it functions at least in part as an ascending cardiovascular pathway.

3. Physiological evidence for a cervical spinal projection to the cerebellum have been confirmed with detailed anatomic studies.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 127 Biological modulation of military performance

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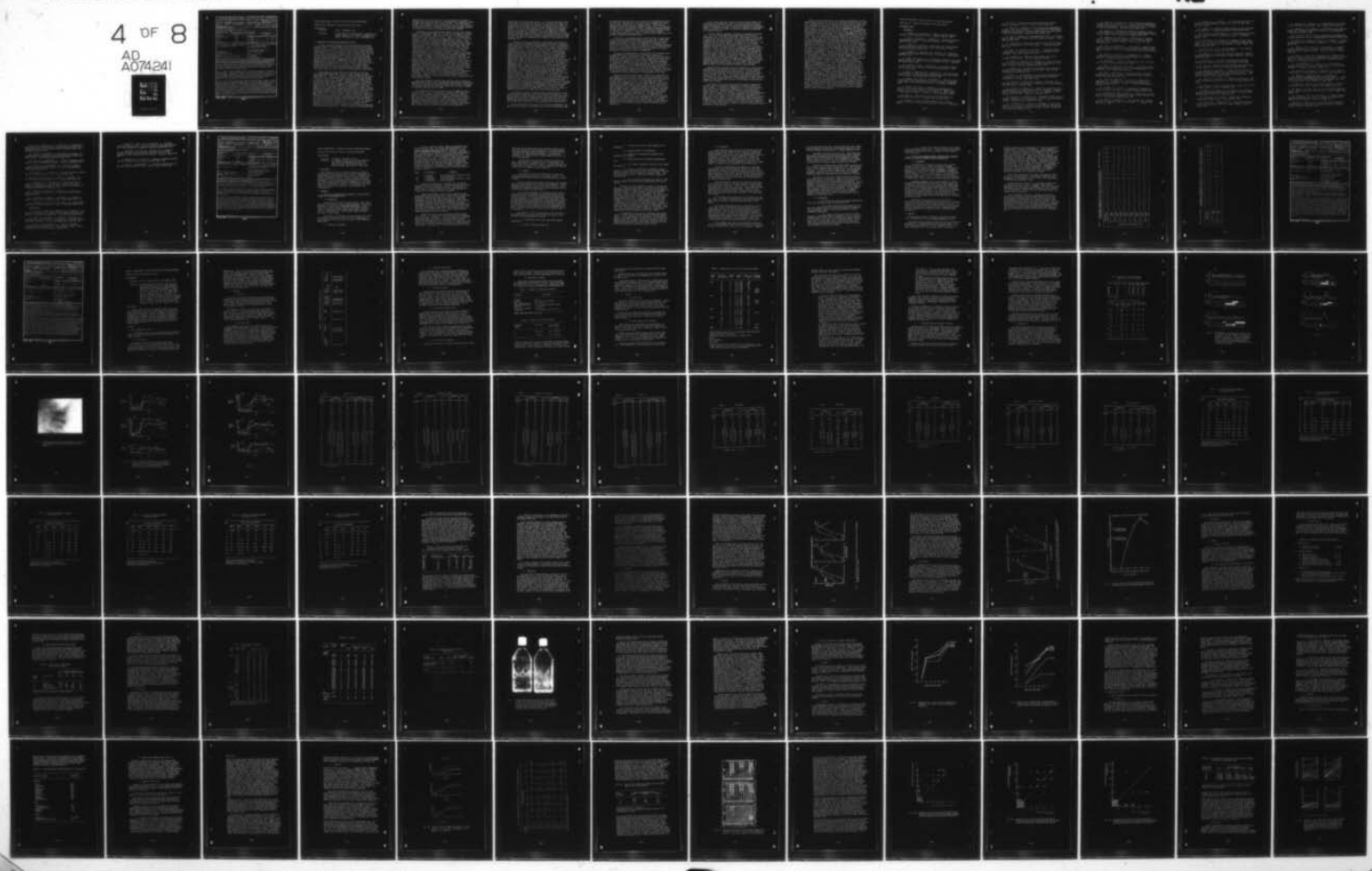
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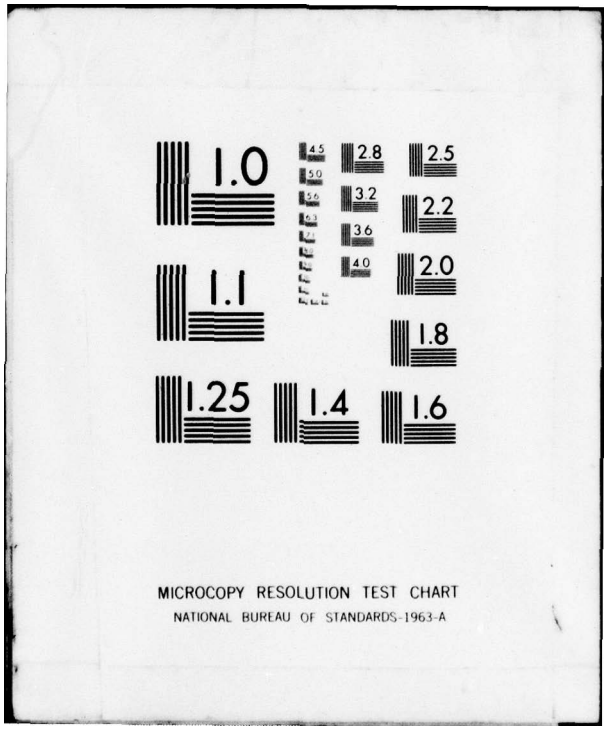
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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMIS--ETC(U)
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6449	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102B S01	00	128
b. CONTRIBUTING				
XXXXXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Mechanism of Response to Military Stress

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 002300 Biochemistry 013400 Psychology

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76 07	CONT	DA	In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	3	309
FISCAL YEAR CURRENT 79	2	246

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) stress (U) cyclic nucleotides (U) neurotransmitters (U) neurochemistry (U) microwave inactivation (U) lateralization of cerebral function

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To examine neurochemical mechanisms regulating neuroendocrine responses involved in adaptation to stress, providing database for interpretation of military field studies and recommendations for prevention and/or treatment of breakdown in soldiers. To examine neurochemical mechanisms mediating lateralization of function, spatial abilities and recovery from cerebral injury.

24. (U) Analysis of role of neurotransmitter pathways in regulation of hormonal response to stress. Effect of stimulation or lesion of specific pathway (i.e., noradrenergic dopaminergic, serotonergic). Effect of stress or centrally-acting hormones on cyclic nucleotides and neurotransmitters in specific brain regions. In-vivo determination permitted by use of microwave enzyme inactivation system designed in this laboratory. Role of dopamine in lateralization of cerebral function.

25. (U) 77 10 - 78 09 Cold exposure produced a marked elevation in cerebellar levels of cyclic guanosine monophosphate (cGMP) whereas the equally potent stressor of forced immobilization had no effect. Locomotor activity also caused marked elevations in cerebellar cGMP, and elevations in six other brain regions out of eighteen studied. It will be important to examine the role of locomotor activity in the neurochemical response to environmental stress. We have used a technique developed in this laboratory to show that while dendrites in substantia nigra contain dopamine, this transmitter is not releasable by potassium depolarization. We have demonstrated a relationship between the ratio of dopamine in left vs right ventral striatum and lateralization in behavioral tasks. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 128 Mechanism of Response to Military Stress

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I. Mechanism of Neuroendocrine Response to Stress

In order to study neurochemical mechanisms determining adaptive hormonal response to environmental stress, we have successfully developed new models. It is well established that stress causes elevations in plasma corticosterone (1-5) and prolactin (5) while lowering plasma growth hormone (3,4). Corticosterone levels following stress vary with time of day, reflecting the circadian rhythm in testing levels (2). It has been demonstrated that habituation occurs to the corticosterone response induced by handling (3,4). Stress is reported to activate central noradrenergic (NE), dopaminergic (DA), and serotonergic (5HT) neurons (6-12). Increases in cAMP levels in brain tissues have been demonstrated following exposure to NE, DA or 5HT in vitro (16,17) and to NE (but only minimally to DA) in vivo (18). It has been recently reported that cold stress elevates cerebellar cyclic GMP (13,14). Cyclic nucleotides are known to activate protein kinases. Rodknight (15) notes a possible stress component in brain protein kinase activity.

The foregoing led us to predict that psychological stressors will elevate cGMP and/or cAMP. An ACTH fragment reportedly lowers brain GABA (19). As ACTH is released in stress and GABA administration lowers cGMP (20), it would seem highly desirable to study the effects of stress (as well as ACTH and other peptides) on brain cGMP and GABA, as well as norepinephrine and cAMP. A method has been established in our laboratory which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5' monophosphate (cAMP), cyclic guanosine 3'5' monophosphate (cGMP) and norepinephrine in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner for cyclic nucleotides (21,22) and the enzymatic method of Graham and Aprison for GABA and GLU (23). To ensure optimal support of the stress studies, we have modified assay procedures to increase the sensitivity of our assay system by acetylating our samples as described by Harper and Brooker (J. of Cyclic Nucleotide Research 1:207-218, 1975). This has increased our sensitivity into the femtomole range. It is thought that cGMP is responsive to cholinergic transmission (24,27) and under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (25-27). Further studies in our laboratory have

demonstrated that the technique of using high-intensity microwave irradiation for enzyme inactivation as indispensable for determining levels of cAMP, cGMP and GABA in brain regions (28,29). The elimination of artifact has permitted accurate assessment of levels of these substances in the regions studied. In addition, for many of the regions, the work is unique in that levels have never previously been reported.

Dinnendahl (30) found that marked elevation of brain cyclic guanosine 3'5' monophosphate (cGMP) was elicited in mice subjected to forced swimming in cold water, exposed to a hot plate, or allowed to fight. These effects were ascribed to "stress". Mao et al (31) noted similar elevation of cGMP in rat brain following exposure to cold. In both of the above reports, the increases in cGMP were most striking in the cerebellum. Because forced immobilization was recognized as a potent stressor (32) we have previously compared the effects of that stressor with the effects of cold exposure on brain regional cGMP, cAMP, and plasma hormonal indices of stress in the rat (33). While a 5 minute cold exposure produced an almost three-fold increase in cerebellar cGMP, five minutes of forced immobilization had no effect. Both procedures however, produced marked increases in plasma corticosterone (CS) and prolactin (Prl), and decreases in plasma growth hormone (GH). Such changes are typical in rats exposed to stressful conditions (1-5, 34,35). It seemed to us that the elevations in cerebellar cGMP might be associated with increased locomotor activity in the cold-exposed group, rather than stress per se. Swimming or fighting clearly involve increased locomotor activity. In the case of the hot plate experiment, the animals were placed on a 56°C surface for 20 seconds. Within 8-10 seconds of exposure to a hot plate test, opiate-naive mice begin jumping, lifting and licking or shaking their paws (36). Hence one might expect that considerable locomotor activity might be engendered during an exposure to this noxious stimulus as prolonged as 20 seconds. It has also been reported that mere cold exposure is sufficient to produce elevations in cerebellar cGMP in the rat (31). Cold exposure, however, is also known to elicit marked increases in locomotor activity (37,38).

Rubin and Ferrendelli (39,40) have noted that physical shaking or administration of drugs causing increased locomotor activity (amphetamine, harmaline, oxotremorine or pentylenetetrazol) are followed by increases in cerebellar cGMP whereas drugs which depress motor activity (diazepam or reserpine) decrease levels of cGMP.

We examined the effect of locomotor activity on cGMP and cAMP in 18 brain regions in rats. We found that cerebellar cGMP levels were twice as high in animals allowed 5 minutes of running in activity wheels as in animals merely allowed to explore the immobilized wheel for 5 minutes. Lesser elevations in cGMP levels were seen in many of the brain regions examined and, similar to the regional pattern of elevation following cold exposure (33), the magnitude of the effect was greatest in the cerebellum and midbrain. No changes were found in cAMP. Plasma levels of hormones indicative of stress were not

significantly different between groups. We completed additional studies on the effect of decreased locomotor activity on brain cyclic GMP. We found immobilization produced a decrease in cGMP which was slight at 5 minutes but a 50% decrease was seen following immobilization lasting 15 minutes. The role of the cerebellum in the coordination of movement has of course, been well established (41). We suggest that locomotor activity contributes to elevations in cerebellar cGMP seen in rats exposed to stressful conditions and may be a contributing factor in changes in cerebellar cGMP levels seen following administration of certain drugs as well. We expect our observations to stimulate re-examination of much published data.

A new area of critical importance to understanding the effects of stress on emotional breakdown is being opened up by studies indicating direct effects of steroid and peptide hormones on brain. Corticosteroids bind preferentially to specific regions in brain, notably brainstem and hippocampus (42,43). The binding is both to cytosol and to nuclei and is influenced by level of circulating corticoids (42). Corticosterone administration causes a marked increase in the activity of tryptophan hydroxylase (the enzyme that synthesizes serotonin) in midbrain (43). These observations have led to speculation that the serotonin neurons which project from the raphe cells to the hippocampus (44,45) might mediate the feedback inhibitory effect of glucocorticoids on the pituitary-adrenal axis. There is controversy over whether or not glucocorticoids depress the single unit firing from raphe cells (46,47). An increase in hippocampal theta is reported to occur 1 hr following glucocorticoid administration (48). Raphe stimulation, which releases 5HT (49), blocks habituation to auditory startle response (50). In one study of glial tumor cells in culture, glucocorticoids doubled the cAMP response to NE but at least a 20 hr exposure to the glucocorticoids was required for minimal effect (51). From the above observation, it appears likely that the direct effect of glucocorticoids on brain is more related to chronic, rather than acute effects of stress. It has been reported (52) that adrenal cortical hormones facilitate the activity of brain adenylate cyclase *in vitro*. Activity is decreased following adrenalectomy and this decrease is reversed by maintenance with dexamethasone or aldosterone. Activity is also decreased by hypophysectomy and this decrease is reversed by either ACTH or dexamethasone. Manipulations which affect adenylate cyclase activity *in vitro* are very dependent on specifics of tissue preparation and may not alter cAMP levels *in vivo*. Hence the effect of endocrine manipulations on cAMP levels must be studied *in vivo*.

We examined the effect of hypophysectomy or adrenalectomy on brain levels of cGMP and cAMP both at rest and following cold exposure. There was no effect of either ablation on resting levels of either nucleotide, and the marked elevation in cGMP following cold exposure was also unaffected by either surgical procedure. A slight effect of saline ingestion on cAMP levels was noted in the hypothalamus and olfactory bulbs.

The primary molecular sites of action of the drugs apomorphine and RO 20-1724 are thought to be different, but the cyclic nucleotides, cAMP

and cGMP have been postulated to be involved in the mechanism of action of both drugs. Apomorphine is considered to be a dopamine (DA) agonist that directly stimulates the DA receptor (53,54). Stimulation of dopamine receptors by dopamine or apomorphine may result in inactivation of dopamine sensitive adenylate cyclases. Dopamine stimulated adenylate cyclases have been demonstrated in many brain regions including caudate, n. accumbens, o. tubercle, s. nigra, and frontal cortex (55,56). In vivo, apomorphine has been shown to increase striatal cAMP levels (57) as well as cerebellar cGMP levels (58).

RO 20-1724, a 3,4-dialkoxybenzyl-2-imidazolidinone, has been shown to be a potent phosphodiesterase inhibitor in vitro (59). RO 20-1724 potentiates elevations in cyclic AMP in guinea pig cortical slices in response to adenosine, histamine, norepinephrine and glutamate (60). RO 20-1724 also increases basal cyclic AMP levels in guinea pig cerebellar slices (61). We were interested in determining whether RO 20-1724 could serve as a pharmacological amplifier for in vivo effects of a neurotransmitter receptor agonist such as apomorphine.

In vivo the phosphodiesterase inhibitor RO 20-1724 increased levels of cyclic AMP in all 4 tested regions: pituitary, cerebellum, n. accumbens-o. tubercle and corpus striatum. The elevation was 12 fold in the pituitary which appears to have a very responsive cyclic AMP system. RO 20-1724 also significantly increased cyclic GMP levels in vivo in n. accumbens-o. tubercle and corpus striatum. Experiments performed in vitro with RO 20-1724 have suggested that the drug is a much more effective inhibitor of cyclic AMP phosphodiesterase activity than cyclic GMP phosphodiesterase activity (59). Our results demonstrate that RO 20-1724 in vivo increases cyclic AMP levels more than cyclic GMP levels in the regions tested. However, the effects on cyclic GMP must also be considered in assessing the biochemical pharmacology of this drug.

Apomorphine alone increased levels of both cyclic nucleotides in some regions, but the increase in cAMP in corpus striatum was not seen when apomorphine injections were preceded by injections of the vehicle used for RO 20-1724. The failure to find elevated cyclic AMP in striatum after apomorphine in this particular experiment could be explained by the somewhat higher "control" cAMP levels in vehicle-saline injected rats vs the saline injected controls. On the other hand in other experiments in our laboratory (unpublished data), we have found that the cAMP response to apomorphine in vivo in striatum is variable and not robust. This has also been observed by other investigators (Michael Schmidt, personal communication). The major responses to apomorphine were increased cyclic GMP levels in cerebellum and n. accumbens-o. tubercle and a very large cyclic AMP increase in the pituitary. DA receptors have been demonstrated in the n. accumbens-o. tubercle but none are found in the cerebellum (62). It has been postulated that the apomorphine-induced cyclic GMP elevation might be mediated via a multi-synaptic pathway originating outside the cerebellum (58).

Since we have shown that locomotor activity elevates cGMP in the cerebellum, and apomorphine is known to stimulate motor activity we are investigating the role of motor activity in mediating the apparent effects of apomorphine on cGMP. Since RO 20-1724 seems to have sedative effects, we expect to find that its effects on cGMP are not mediated via locomotor activity.

Pituitary cAMP was remarkably responsive to apomorphine or RO 20-1724. Levels of cyclic AMP increased approximately 8 fold after apomorphine, and 12 fold after RO 20-1724. The turnover of cyclic AMP in the pituitary must be relatively high and the system very sensitive to demonstrate such large increases. We find that the effects of apomorphine and RO 20-1724 appear to be additive in the pituitary. The pituitary has been shown to contain DA receptors and it has been suggested that dopamine may act directly on the pituitary to inhibit prolactin release. The administration of apomorphine in vivo decreases prolactin. Schmidt and Hill recently reported that adenylate cyclase activity in intact pituitaries in vitro was unaffected by apomorphine although striatal adenylate cyclase activity was stimulated under the same experimental condition. Our results, however, show that in vivo pituitary cAMP is elevated by apomorphine. Either apomorphine is able stimulate pituitary DA receptors in vivo but not in vitro, or possibly apomorphine affects pituitary cyclic AMP through activation of a system originating outside the pituitary. Our data suggest that RO 20-1724 inhibits phosphodiesterase activity in vivo in rat, increasing both cAMP and cGMP in brain and markedly increasing pituitary cAMP.

Recent work has supported a role for asymmetries in central nervous system dopamine (DA) in spatial preference in rats and has suggested that the relevant asymmetries are nigro-striatal. A critical piece of evidence supporting the role of nigro-striatal dopaminergic asymmetries in spatial preference in intact animals has been the study of Zimmerberg et al (73). These investigators reported that side preference in a T-maze shock-escape apparatus is associated with a higher level of dopamine in the contralateral striatum. We attempted to replicate and extend their findings.

Our results are in accord with the body of evidence supporting a role for intrinsic dopaminergic asymmetries in spatial preference in intact animals. In contrast to Zimmerberg et al, we found no evidence that nigro-striatal dopaminergic asymmetries were correlated with spatial preference. We have, however, found an association between higher levels of dopamine in the nucleus accumbens and olfactory tubercle on one side of the brain and a greater tendency to turn toward that side. Our results support a role for dopaminergic asymmetries in spatial preference, and in contrast to previous work suggest that dopaminergic asymmetries in the nucleus accumbens and olfactory tubercle, rather than in the striatum, underly turning preference, and hence focus attention of the meso-limbic dopaminergic system as a possible substratum of spatial preference in intact animals.

There is ample evidence that stress releases catecholamines from the adrenal medulla and by some indications, from brain neurons as well (6-12, 63). Although neuronal firing rates are one determinant of release (64), local presynaptic receptors are reported to influence releasability of brain NE (65). The latter work was done with exogenous, labelled NE. Such a model could prove misleading in that numerous storage compartments may be labelled, and therefore, specificity limited. Our laboratory has developed the first in vitro model of release of endogenous NE from brain tissue (66). This advance permits us to examine the effects of acute and chronic stress or injury on pre-synaptic modulation of transmitter release. Such studies would not have been feasible without the development of our in vitro model. We have previously measured the release of NE in vitro from hypothalamus, a region with a high NE concentration, in response to depolarizing concentrations of KCl, (66). We also reported that DA was released from striatum, a region rich in DA, after incubation with KCl (67). We have recently shown that a portion of the DA release in corpus striatum may be calcium-independent (68) in marked contrast to NE release from hypothalamus. The striatum, accumbens-tubercle and hypothalamus are dopaminergic terminal areas, while the s. nigra contains DA cell bodies and dendrites. Release has been thought to occur primarily from DA terminals. However recent studies have suggested a role for s. nigra dendritic DA release (69,70). Release of ^{3}HDA from s. nigra by KCl has been reported in vitro (71) and in vivo (72). Although we also have observed release of previously taken up ^{3}HDA by elevated KCl (unpublished results), we were unable to demonstrate KCl stimulated release of endogenous DA from s. nigra in several experiments. Since ^{3}HDA can be taken up by NE terminals, perhaps the observed release of ^{3}HDA by KCl is from NE terminal areas rather than from DA dendrites. We found that both KCl and amphetamine released endogenous NE from the substantia nigra. d-Amphetamine did release endogenous DA from the s. nigra preparation. Possibly, amphetamine and KCl release endogenous DA from different pools. KCl may be an effective releaser only from terminals while amphetamine could also release from the dendrite or cell body DA stores. We have suggested (66-68) that release of endogenous catecholamines might not always parallel the release observed during previously taken up radiolabeled amine. The present data with regard to s. nigra DA release demonstrate the utility of the endogenous release model. d-Amphetamine stimulated both NE and DA release from regions throughout the rat brain. This widespread release of both transmitters should be considered in attributing the behavioral effects of amphetamine to specific neuronal systems.

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7. Kant, G.J., and Meyerhoff, J.L. Release of endogenous NE and DA from rat brain regions in vitro. Life Sci. 23:2111-2118, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6440	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISR'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	129
b. CONTRIBUTING				
c. ~~CONCURRENT~~	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Parasitic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING	78	3.0	415
FISCAL YEAR — CURRENT	79	3.0	157

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Parasite; (U) Schistosomiasis; (U) Malaria; (U) Primate; (U) Trypanosomiasis; (U) Leishmaniasis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To study physiological, biochemical, pathological and epidemiological aspects of parasitic diseases of military importance. To evaluate existing techniques and to develop new techniques for diagnosis, prevention, treatment and control.

24. (U) Culture systems and animal models of parasitic diseases will be developed and used to study the parasites of interest, the parasitic disease process, and the effectiveness of new diagnostic, preventive and therapeutic measures. Studies will emphasize but will not be restricted to malaria, leishmaniasis, trypanosomiasis, and schistosomiasis.

25. (U) 77 10-78 09 A primate model of African trypanosomiasis was developed using cynomolgous monkeys infected with the Wellcome strain of T. rhodesiense. This strain produced more severe pathological changes in the cynomolgous monkey than in rhesus. A candidate antitrypanosomal drug (WR 119290) with outstanding activity in the mouse model, failed to cure infections in the cynomolgous monkey. The Khartoum strain of Leishmania donovani produced a uniformly lethal infection in cynomolgous monkeys which resembled human visceral leishmaniasis. The HM (Mediterranean) strain of Leishmania donovani produced only a mild, transient disease which self cured in 3 of 4 cynomolgous monkeys. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 129 Parasitic Diseases of Military Importance

Investigators.

Principal: LTC David E. Davidson, Jr., VC
Associates: LTC Anthony Johnson, VC; MAJ Larry Hendricks, MSC; MAJ Bryce Reddington, MSC; MAJ Lyle Ketterling, MSC; MAJ Michael Reardon, VC; MAJ Ralph Bunte, MSC; MAJ Ray H. Perry, MSC.

1. Description.

Basic Research Studies on Parasitic Diseases of Military Importance are being conducted with the goal of developing improved techniques and new knowledge to assist in diagnosis, prevention, treatment and control. Parasite culture systems and animal models of the parasitic diseases of interest are being developed to study the physiology and biochemistry of the parasite, and to study the host-parasite relationship and the pathogenesis of these diseases. Emphasis is placed upon, but is not restricted to malaria, leishmaniasis, schistosomiasis and trypanosomiasis.

2. Progress.

a. Use of the Cynomolgous Monkey Model for the Testing of Candidate Antitrypanosomal Drugs.

1.) Background

Previous drug efficacy studies conducted in this laboratory have utilized rhesus monkeys (Macaca mulatta) infected with Wellcome CT or 1886 strain Trypanosoma rhodesiense. A small 4-monkey pilot study conducted last year utilizing Wellcome CT strain in cynomolgous monkeys (Macaca fascicularis) suggested that this alternative host, which is more readily available, would be a suitable model.

WR 119,290, an experimental antitrypanosomal drug, which had exhibited outstanding activity against T. rhodesiense in mouse screening tests was selected for study in the cynomolgous monkey model.

2.) Materials and Methods

Fourteen cynomolgous monkeys (Macaca fascicularis) were utilized in this study. Ten monkeys were inoculated I.V. with 50,000 Trypanosoma rhodesiense organisms (Wellcome CT strain) obtained from donor mice and purified on a column of DEAE sephadex. Treatment with WR 199,290 was initiated when parasitemia was evident in all monkeys. Eight infected monkeys were given WR 119,290 daily for 3 days by nasogastric intubation at 30 mg/kg per day on days 4, 5 and 6. Two infected and two uninfected monkeys were sham-treated with 5 ml of drug vehicle (physiologic saline) for 3 days. The two remaining uninfected monkeys were given WR 119,290 in the same dose and regimen (30 mg/kg x 3 days) as the infected group. All monkeys, including controls, were fasted for 18 hours before and 2 hours after each administration of drug or vehicle.

Group	No. of Monkeys	Treatment
I	8 Infected	WR 119,290 orally 30 mg/kg x 3 days
II	2 Infected	Vehicle Controls
III	2 Uninfected	WR 119,290 orally 30 mg/kg x 3 days
IV	2 Uninfected	Vehicle Controls

Monkeys were immobilized for handling by intramuscular injection of 0.2-0.3 ml Ketamine. Blood was obtained for parasite counting by pricking the marginal ear vein or footpad with a sterile lancet. Giemsa stained blood films were prepared daily on days 0-10; twice weekly or weekly thereafter.

General observations of the physical condition of the animals were made and recorded daily. Body weight was determined and blood collected three times prior to day 0, twice weekly between day 0 and 28, and weekly through day 49. Hematologic measurements included RBC, hematocrit, hemoglobin, mean corpuscular volume, platelets, erythrocyte sedimentation rate, WBC, differential and reticulocyte counts. Biochemical determinations included blood glucose, serum protein, serum albumin, SGOT, SGPT, LDH, alkaline phosphatase, BUN, creatinine, total bilirubin, and serum sodium potassium and chlorides. Rectal temperature was determined whenever blood was collected.

Monkeys surviving to day 49 (excepting Group IV) were sacrificed by I.V. injection of Lethane and gross and histopathologic examinations performed. At sacrifice samples of spleen and pooled prescapular and axillary lymph nodes were collected aseptically and homogenized for inoculation into groups of 5 mice each in order to detect subpatent parasitemia or sequestration of trypanosomes. Blood was also collected for subinoculation during

the course of the study (after drug treatment) whenever parasitemia was subpatent by smear examination. Selected smear-positive blood was subinoculated periodically as a control for the validity of the subinoculation procedure. Each mouse was inoculated I.P. with 0.2-0.4 ml of whole blood or 0.2-0.4 ml of 20% tissue homogenate.

Blood films of the recipient mice were followed for 15 days. The schedule of treatment, blood film examination, and blood collection is presented in Table 1. At each bleeding, 5 ml of blood was collected; 2 ml in EDTA for hematology and subinoculation; and 3 ml clotted for serum chemistry.

3.) Results

The results of blood film examinations and blood and tissue subinoculations into mice are presented in Table 1. All inoculated monkeys become patent by blood film examination within 2-3 days.

The two vehicle-treated controls (A-09 and A-26) remained smear-positive through day 5, and then became subpatent. Patency was again noted in A-26 on day 9, and in A-09 on day 28. During the period of subpatency, both vehicle controls were positive by subinoculation except for A-09 on day 7.

All of the drug treated, infected group became subpatent on the day after drug treatment was initiated (one day earlier than the controls). During the subpatent period following the initial parasitemia, all 8 monkeys exhibited parasitemia either by film examination or subinoculation. One treated monkey (A-48) remained negative throughout the post-treatment period. Monkey A-14 was also negative throughout the post-treatment period with the exception of a single positive result from subinoculation on day 10. Monkey A-33 also exhibited a mild post-treatment course, with a positive subinoculation on day 10 and a positive blood film on day 17.

Characteristic signs of trypanosomiasis were observed in the infected vehicle control monkeys, and in at least 5 of the infected, drug treated monkeys as follows:

a.) Intermittent fever (with associated lethargy, inappetence).

b.) Mild to moderate weight loss.

c.) Progressive decrease in RBC, hematocrit and hemoglobin.

d.) Marked increase in reticulocytes.

e.) Reduction in total WBC and platelets (not observed in all monkeys).

f.) Marked increase in erythrocyte sedimentation rate.

g.) An increase in monocytes during the early stages of infection.

h.) Evidence of neurologic involvement, perhaps related to trypanosome invasion of the central nervous system was noted clinically in only one monkey (the control, A-26). This was first noted on day 43. Signs were limited to tremors and incoordination.

i.) Only one monkey died during the course of study. This was monkey A-30 from the drug treated group which died on day 58.

The most consistent biochemical finding in infected monkeys was a progressive and dramatic increase in serum alkaline phosphatase, beginning about day 7, and persisting throughout the course of infection. Slight increases in SGOT and SGPT were observed in several infected monkeys between days 3 and 14, returning to within normal limits thereafter. LDH values varied over a wide range in both infected and control monkeys, making interpretation difficult. It appears that LDH may have been elevated in several of the infected monkeys late in the course of infection. Serum albumin decreased progressively in many infected monkeys, particularly in the later stages. Abnormal increases in BUN and creatinine were noted in a few monkeys, and these were limited to the advanced stages of the disease. Serum glucose was also decreased late in infection. Serum sodium, potassium and chlorides remained within normal limits.

No abnormalities were noted in uninfected drug-treated controls. Since drug was administered during Ketamine immobilization, clinical observations are unreliable. No vomiting was noted, nor any other untoward clinical sign during or after drug administration in any monkey. Hematologic parameters and biochemical determinations were within normal limits for all uninfected drug-treated and uninfected vehicle controls.

4.) Discussion

Blood parasite levels attained in the present study were disappointing, and the tendency of the infection to remain subpatent throughout much of the course of infection complicated interpretation of drug effects, and necessitated heavy reliance on subinoculation techniques to detect infection. Perhaps the cynomolgous monkey is less susceptible to infection with this strain of trypanosome than the rhesus monkey, or perhaps the cynomolgous monkey has a greater tendency to sequester its trypanosomes. A parallel study is needed to clarify this point.

Erythrocyte sedimentation rate appears to be the most sensitive and consistent hematological indicator of infection, although RBC, hematocrit and hemoglobin were also rather consistent indicators. Reticulocytosis regularly paralleled the anemia. Rectal temperature, body weight, WBC and platelet counts were also useful indicators of clinical condition. The monocytosis which was observed early in the course of infection in most monkeys is an interesting observation.

Alkaline phosphatase was the most significant and consistent indicator of infection, becoming elevated at about 7 days, and progressively increasing. Slight increases in SGOT and SGPT were noted early in infection (days 3-14). Serum albumin decreased progressively in most infected monkeys. LDH levels were erratic and not particularly useful. Abnormalities in glucose, BUN, creatinine and bilirubin were noted only in the late stages of infection.

WR 119,290 was well tolerated orally at 30 mg/kg per day for 3 days. No clinical, hematologic nor biochemical abnormality which could be related to drug intolerance was noted in any monkey.

WR 119,290, administered orally at 30 mg/kg per day for 3 days (days 4-6 post-inoculation) influenced the course of trypanosome infection, but was relatively ineffective in curing infected animals. All of 8 treated monkeys became subpatent within 24 hours after initiation of drug treatment (one day before vehicle-treated controls became subpatent). All of 8 drug treated monkeys were subpatent and subinoculation negative on day 7 (the day after termination of drug treatment). One of two vehicle-treated controls was subinoculation positive on day 7.

Post-treatment parasitemia and subinoculation data for 5 of the 8 treated monkeys clearly indicate failure of the drug to cure the infection (A-02, A-08, A-12, A-13, and A-30). Hematolo-

gic and serum biochemical data, and body weight and rectal temperature observations indicate the uninterrupted progression of the clinical syndrome of trypanosomiasis in these 5 monkeys as well.

One monkey (A-48) may have been cured. After drug treatment, blood films and subinoculations were consistently negative. Hematology and biochemistry after drug treatment were entirely normal, except for an increase in rectal temperature of unknown etiology observed on day 49. Due to a laboratory error, terminal subinoculations of spleen and lymph node were not performed.

Except for a single positive subinoculation on day 10, all other indicators suggest that A-14 was also cured. RBC and hematocrit in this monkey decreased through day 17, and then appeared to recover. All other clinical, hematological, and biochemical parameters remained within normal limits after drug treatment. Spleen and lymph node subinoculations were negative at necropsy.

The course of infection in monkey A-33 was also significantly modified by drug treatment. Blood in this monkey was subinoculation positive on day 10, and smear positive on day 17. Otherwise, blood films and subinoculations were negative. RBC and hematocrit were decreased on days 7-17, and recovered thereafter. Increased in sedimentation rate and reticulocyte count noted on days 3-10 were reversed after drug treatment. All other hematologic parameters remained within normal limits. Biochemical parameters were within normal limits after drug treatment. Terminal subinoculations of spleen and lymph node were not performed due to laboratory error.

5.) Conclusions

a.) WR 119,290 was well tolerated when administered orally for 3 consecutive days at 30 mg/kg per day.

b.) WR 119,290 exhibited antitrypanosomal activity when administered orally for 3 consecutive days at 30 mg/kg per day to cynomolgous monkeys infected with Wellcome CT strain Trypanosoma rhodesiense.

c.) The tendency of Wellcome CT strain to remain subpatent in cynomolgous monkeys throughout much of the course of infection makes it difficult to interpret the curative activity of WR 119,290 in this study. One of 8 monkeys appears to have been cured. Two additional monkeys appear also to have been cured although trypanosomes were observed in blood 3 to 10 days after termination of the drug regimen.

d.) It seems likely that WR 119,290 will be tolerated at considerably higher doses. A second efficacy study is being considered to evaluate the curative potential of WR 119,290 at higher drug doses.

b. The Use of Cynomolgous Monkeys Infected with Leishmania donovani as a Model of Human Visceral Leishmaniasis.

1.) Background

Leishmaniasis is a parasitic disease of the reticuloendothelial system which is widely distributed throughout tropical and semi-tropical regions of the world. Transmission is by the bite of tiny sandflies (genus Phlebotomus or Lutzmyia). Depending on the species and strain of Leishmania involved, the disease appears in one of 3 clinical forms: visceral, mucocutaneous or cutaneous. All forms of leishmaniasis are capable of producing large numbers of cases in military personnel operating in endemic areas, and may result in acute or long-term casualties.

Leishmania donovani, the causative organism of visceral leishmaniasis, has been extensively used as a model organism in leishmanial research; and, for drug screening, the hamster model is utilized by the WRAIR Program (Stauber Method as modified by Hanson). While this hamster model has proven to be a sensitive and reproducible screening model, it is considered that compounds showing antileishmanial activity in this system need to be evaluated in another animal model, preferably one more closely resembling the human disease, before such compounds are considered for clinical trials.

Accordingly, investigations has been initiated to characterize the clinical and pathological features of L. donovani infection in cynomolgous monkeys, and to select a strain or strains which can be used in a primate model for preclinical evaluation of candidate antileishmanial drugs. Cynomolgous monkeys have been selected for use because of their relatively good availability.

3. Results.

Two experiments have been conducted in Cynomolgous monkeys. First, the Khartoum strain of Leishmania donovani was used.

Five monkeys were inoculated intravenously with 40-100 million amastigotes of L. donovani (Khartoum) per kg body weight. All inoculated monkeys developed a clinical syndrome similar to visceral leishmaniasis as it is observed in man. The initial

clinical sign usually was splenomegaly in 3-7 weeks post-exposure followed shortly by onset of pancytopenia. Typical changes included a drop in platelet count from 5,000,000 to 100,000 per cmm or less, a decrease in hematocrit from 32-40% to 7-17%, a drop in total leucocyte count from 5-10,000 to 1-3,000 per cmm, and weight loss averaging 15%. Animals usually died 6-20 weeks after inoculation. At necropsy, spleens and livers from infected monkeys averaged 8.5 and 2.5 times heavier, respectively, than those organs from non-infected control animals. Parasites were seen microscopically in liver, spleen, and bone marrow at necropsy, and those parasites could be recovered in culture. Histopathologic lesions included severe, diffuse granulomatous inflammation of the spleen, liver, and abdominal lymph nodes. Granulomatous interstitial pneumonia and epididymitis also were evident. Meningeal hemorrhages were occasionally observed.

These results suggest that this monkey model may be suitable for use in laboratory studies of the biology, immunology, and pathogenesis of leishmaniasis, as well as for use in the testing of candidate antileishmanial drugs.

A second study was conducted in 4 Cynomolgous monkeys using the Mediterranean HM strain of L. donovani. Again, 40-100 amastigotes per kg body weight from hamster livers were inoculated intravenously, and the monkeys were followed by clinical observation, by hematology, clinical chemistry, bone marrow biopsy, and terminal parasite subculture.

In this experiment, HM strain L. donovani produced only a mild and self-limiting clinical disease. Three of the 4 inoculated monkeys self-cured within the 7 week observation period, and were negative for parasites by subculture of spleen, liver, bone marrow, and lymph nodes at the time of sacrifice. The fourth monkey was parasite positive at necropsy, although organ changes were minimal. It appears that the Cynomolgous monkey may not be very susceptible to this hamster-adapted strain, and that this host-parasite combination may not lend itself well to laboratory use.

Table 1. *Trypanosoma rhodesiense* Infections in Cynomolgous Monkeys: Blood Film and Mouse Subinoculation Data for Drug Treated and Untreated Monkeys.

	Days Post-Inoculation		1	2	3	4	5	6	7	8	9	10	14	17	21	28	35	43	49
Drug Treated Infected Monkeys	A-02	Smear	-	+	<1	1	-	-	-	-	<1	<1	2	-		+	+		
		Inoc.							-			+	+		+				
	A-08	Smear	-	+	<1	+	-	-	-	-	<1	<1	+	-		+	-		
		Inoc.							-			+	+		+				
	A-12	Smear	-	-	+	<1	-	-	-	-	-	-	-	-		-	+		
		Inoc.							-			+	+		-				
	A-13	Smear	1	+	<1	+	-	-	-	-	-	-	<1	-		+	+		
		Inoc.							-			+	+		+				
	A-14	Smear	-	-	+	1	-	-	-	-	-	-	-	-		-	-	-	-
		Inoc.							-			+	-		-				
	A-30	Smear	-	-	+	3	-	-	-	-	-	-	<1	+		-	+		
		Inoc.							-			+	+		+				
	A-33	Smear	-	+	<1	<1	-	-	-	-	-	-	-	+		-	-	-	-
		Inoc.							-			+	-		-				
	A-48	Smear	-	+	<1	5	-	-	-	-	-	-	-	-		-	-	-	-
		Inoc.							-			-	-	-					

Table 1 (continued). Trypanosoma rhodesiense Infections in Cynomolgous Monkeys: Blood Film and Mouse Subinoculation Data for Drug Treated and Untreated Monkeys.

	Days Post-Inoculation		1	2	3	4	5	6	7	8	9	10	14	17	21	28	35	43	49
Controls	A-09	Smear	-	+	+	-	+	-	-	-	-	-	-	-		+	+	+	+
		Inoc.							-			+	+		+				
	A-26	Smear	-	+	<1	12	2	-	-	-	<1	<1	<1	-		+	+	+	+
		Inoc.							+			+	+		+				

CDC

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	130
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Viral Infections of Man

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 78	4.5	477
CURRENT FISCAL YEAR 79	4.5	479

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: DALRYMPLE, Dr. Joel M.

NAME: BRANDT, Dr. Walter E.

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 77 10 - 78 09 Adenovirus. A microtiter neutralization test for adenovirus antibody was developed using transformed human embryonic kidney cells. The assay proved to be of equal specificity to previous assay techniques and superior to them in terms of sensitivity, manpower required and cost. An enzyme linked immunosorbent assay (ELISA) was technically more difficult and less specific than the microtiter neutralization test. Adenovirus type 21 was the predominant cause of acute respiratory disease (ARD) in basic combat trainees while type 4 and 7 vaccines were administered. Disease rates stayed below 4.0/100/week except in January and February when influenza was also being transmitted. Influenza. Influenza A/USSR/77 (H1N1) caused shortlived outbreaks of mild disease on many military posts in 1977-78. H1N1 isolates were recovered from the US Army Military Academy and the Aberdeen Proving Ground in February. H3N2 isolates were recovered from Ft. Eustis and Ft. Wood in December and January. For Technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 to 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	130
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Viral Infections of Man

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

0026 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING		
FISCAL YEAR CURRENT		

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

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NAME:* Walter Reed Army Institute of Research
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: DALRYMPLE, Dr. Joel M.

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21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 77 10 - 78 09 Arboviruses. Six (6) volunteers were inoculated with over 100,000 plaque forming units of a live attenuated dengue type 2 virus. Five recipients developed viremia, but virus was not recovered from peripheral blood leukocytes as it is in cases of dengue disease. Complement fixing (CF) antibody to soluble crude antigens was not detected as it is in classical dengue fever; CF antibody was detected by reaction with structural (virion) antigens. Volunteers inoculated most recently with attenuated yellow fever virus, serologically related to dengue, developed the lowest dengue neutralizing antibody titers. The serological conversions were broadly cross-reactive because of previous exposure to the flavivirus group antigen carried by the yellow fever vaccine. The vaccine virus appears stable in vivo and safe to use for additional testing. Personnel continued to serve as consultants for the Pan American Health Organization as dengue epidemics spread about the Caribbean region. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 77 to 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 130, Viral Infections of Man

Investigators:

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Description

To define the etiology and ecology of human virus infections particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission to men, other vertebrates and invertebrates, and their survival in nature.

Progress

I. The arthropod-borne viruses

A. Immunization with Live Attenuated Dengue Virus Vaccines Study #1. Safety and Immunogenicity of DEN-2 (PR-159/S-1) in Adult Volunteers.

1. Background

The WRAIR has developed an attenuated dengue type 2 virus, PR-159 (S-1) as a candidate live virus vaccine. The selection, characterization and testing of the S-1 virus strain has been documented in previous annual progress reports. The parent virus, PR-159, was isolated from a patient with benign

dengue fever. The S-1 strain was obtained by clonal selection of a small plaque variant in primary monkey kidney cell culture and subsequent serial passage in diploid fetal rhesus lung cells DBS-FRhL-2. The vaccine virus is temperature sensitive and less virulent for mice by the intracerebral route than the parent virus. It has a markedly reduced ability to cause viremia in rhesus monkeys and chimpanzees compared to the parent virus and produced no CNS disease or inflammatory lesion in rhesus monkeys when injected intracerebrally. During the past year, the vaccine strain has been used in its first human trial.

2. Methods

a. Volunteers

Six adult males who were knowledgeable of microbiological research by training and experience volunteered for the study. All volunteers were thoroughly briefed on the procedure and the risks of the study and each signed a consent form. Each person passed a complete physical examination prior to the initiation of the study.

All volunteers had received yellow fever vaccinations in the past (Table 1). Four men had previously resided for 3 years or more in countries with endemic dengue and one person, G.R., in retrospect, had a history of a dengue type 4 infection in 1967. None of the volunteers had detectable PRNT antibody to the prototype strains of dengue types 1, 2, 3 and 4 at a serum dilution of 1:10.

b. Place and Time

The volunteers were housed on a closed research ward at the United States Army Research Institute of Infectious Diseases (USAMRIID), Ft. Detrick, MD, in two groups. The first two recipients (Group A) were vaccinated on 20 February 1978; the next recipients (Group B) on 3 April 1978. Volunteers entered protective isolation 48-72 hrs prior to vaccination and remained there for 10 days post-vaccination. Volunteers were restricted to the ward and a fenced outdoor courtyard during the period of isolation. All visitors to the isolation area wore face masks to reduce the risk of transmission of respiratory viruses to the volunteers.

Table 1. Volunteers for Den-2 Vaccine, Study #1.

Volunteer Group	Volunteer	Sex	Age Yr	Most recent Yellow Fever Vaccination	Other Flavivirus Infections	Den-2 Vaccination
A	F.H.T.	M	41	May 1976	none	Feb 78
A	W.L.O.	M	43	Oct 1961	none	Feb 78
B	G.R.	M	50	Jul 1969	"Den-4",1967	Apr 78
B	W.H.B.	M	39	Oct 1968	none	Apr 78
B	D.D.B.	M	32	Apr 1974	none	Apr 78
B	M.A.U.	M	28	Jan 1978	none	Apr 78

c. Prequarantine Studies

Each volunteer had a complete physical examination including a chest xray and an electrocardiogram. Preliminary blood studies included CBC, platelet count, prothombin time, activated partial thromboplastin time, thrombin time, BUN, creatinine, SGOT, SGPT, LDH, and C3 level. Values were normal for each volunteer. Urinalysis and urinary urobilinogen concentrations were also normal. In addition, the volunteers had no detectable hepatitis B surface antigen or hepatitis B surface antibody when tested by radioimmunoassay.

d. Follow-up Studies

Each volunteer was examined daily by a physician for 10 days following vaccination and at least twice a week thereafter until Day 21. Oral temperatures were taken 3 times a day for at least 10 days after vaccination and at least once a day thereafter. Blood pressure were measured daily. Blood samples were drawn daily to Day 21 and, later, on Days 30 and 60 for studies of viremia, serological responses and any changes in complete blood counts or serum biochemistries (Table 2). Urinalyses were done on even numbered study days.

e. Vaccination Administration

The vaccine used in this study was a live, attenuated dengue type 2 virus designated DEN-2 (PR-159/S-1) Lot No. 1 (Mfg date Jan 76) prepared by the Department of Biologics Research, Walter Reed Army Institute of Research. The parent virus was isolated in 1969 from a patient with dengue in Puerto Rico. The vaccine strain has two useful markers of attenuation in vitro; uniform small plaque morphology in LLC-MK_2 cell culture and inhibition of growth at 39.3C (temperature sensitivity).

Vials of vaccine were selected at random and transferred on wet ice to the isolation ward at USAMRIID before resuspension in 3.0 ml of sterile water for injection. Each volunteer received 0.5 ml subcutaneously in the upper arm on the morning of Day 0. The unused portion of vaccine was immediately placed on wet ice and transported to WRAIR for immediate virus titration.

f. Vaccine Virus Titration

Samples of vaccine were titrated by plaque assay in LLC-MK_2

cells at 35°C. Vaccine assays were initiated within 3 hours after the vaccination. The observed virus titers (Table 3) were similar to those previously determined for this vaccine.

g. Detection of Viremia

Daily plasma samples were assayed directly on LLC-MK_2 cell monolayers in 25 cm^2 plastic flasks. Three flasks were inoculated with 0.2 ml of plasma each and incubated at 35°C for 1-2 hours to allow virus adsorption. After washing, two

Table 2. Schedule of Laboratory Specimens

Procedure	Days Tested
Viremia	0 to 21
Serology	0,4,7,10,14,17,21,30,60
CBC, platelet count	-3, 0 to 21
Serum Biochemistries*	-3,0,2,4,6,8,10,12,14,16,18,20
Serum C_3	-3, 0 to 21
Fibrin split products	-3, 0 to 21
Urinalysis	-3,0,2,4,6,8,10,12,14,16,18,20

*SGOT, SGPT, LDH, BUN, Creatinine

Table 3. Dengue 2 Virus Vaccine Doses

Volunteer Group	Date Used	Virus Titer/ 0.5 ml dose
A	20 Feb 78	4.5×10^5 pfu
B	3 Apr 78	2.5×10^5 pfu

flasks received fresh nutrient overlay medium for direct plaque assay; one flask was incubated at a permissive temperature (35°C) and the other at a nonpermissive temeprature (39.3°C) for the vaccine virus. After incubation for 14 days, the flasks received

a second overlay containing neutral red stain and the plaques were counted.

The third flask was incubated with liquid nutrient medium at 35°C for 14 days before the supernate was tested for virus by plaque assay.

Circulating monocytes collected daily in heparinized blood were separated and concentrated in Ficoll-Hypaque gradients and incubated in 25 cm^2 plastic flasks for 3 hours at 35°C. After washing, the adherent cells were maintained in fresh medium for 5 days. Daily aliquots of each monocyte culture were tested for virus by plaque assay. The nonadherent cells were cultured for 5 days and then tested for virus by plaque assay.

3. Results

a. Virus Isolation

Viremia was detected in five of the six volunteers. Twenty-four (24) virus isolates were recovered from plasma; only one was obtained by direct plaque assay (Table 4). All virus isolates formed uniform small plaques. None of the viruses tested grew at 39.3°C. No virus isolates were obtained from the monocyte cultures.

The periods of viremia began 8 days (one volunteer) and 9 days (4 volunteers) following immunization and lasted from one to 10 days. The latest virus isolate was recovered on Day 18 from W.L.O.

b. Clinical Response to Vaccination

Each volunteer was interviewed and examined daily by a physician until Day 10 and then at least twice a week until Day 21. Daily observations were recorded on a check list and in written progress notes.

Before vaccination, MAU was found to have a small lymph node in the left axilla which disappeared by Day 0. He was immunized in the right arm. WHB experienced a sore throat from Day -1 to +3. The other volunteers had no complaints or notable physical findings before immunization.

Only one volunteer reported discomfort at the injection site. WLO complained of mild localized pruritus with focal

Table 4. Characteristics of Virus Isolates from Plasma

Volunteer	Day Post-Vaccination	Plaque Size	Virus[1] Titer at 35 C	Virus[1] Titer at 39.3 C	Identity on PRNT[2]
FHT	8	S[3]	6.2×10^2	ND[4]	
	9	S	8.6×10^3	ND	
	10	S	5.1×10^3	ND	
	11	S	3.1×10^4	ND	
	12	S	4.4×10^2	0[5]	DEN-2
	13	S	6.9×10^2	0	DEN-2
	14	S	5.8×10^2	0	DEN-2
WLO	9	S	1.1×10^1	ND	
	10	S	1.2×10^3	ND	
	11	S	6.1×10^3	ND	
	12	S	1.0×10^2	0	DEN-2
	13	S	1.2×10^1	0	DEN-2
	14	S	2.6×10^2	ND	
	18	S	1.4×10^2	0	
WHB	9	S	3.4×10^3	0	
	11	S	2.1×10^3	0	
	13	S	4.6×10^3	0	DEN-2
	14	S	6.3×10^2	0	
DDB	9	S	2.5×10^4	0	
	11[6]	S	1.3×10^4	0	
	11	S	1.5×10^4	0	
	12	S	5.3×10^4	0	
	13	S	4.0×10^3	0	DEN-2
MAU	9	S	1.6×10^4	0	DEN-2

[1] plaque forming units/0.2 ml of supernatant from 14-day LLC-MK_2 culture with liquid medium

[2] PRNT used antibody to New Guinea C, Dengue type 2

[3] small

[4] not determined

[5] no growth

[6] Virus isolate by direct plaque assay in LLC-MK_2 cells. All other isolates were made by plaque assay of supernatant fluid from 14-day cell cultures under liquid medium.

erythema from Day 1 to 3. None of the vaccinees developed regional lymph node enlargement.

Four volunteers developed symptoms during the period of viremia which might be attributed to dengue virus infection. The complaints included headache (3), oral temperature ≥100F (2), myalgia (2), fatigue (2), photophobia (2), eye pain (1), and rash (1). MAU developed the highest temperature (101.8F). Each of the five viremic volunteers had a decline in leukocyte count after the onset of viremia; four individuals had a WBC count of less than 4000 and were considered leukopenic. The temporal relationships between viremia, fever and leukopenia are illustrated in Figure 1. The response of each volunteer was the following.

1. FHT was viremic from Day 8 to 14 but remained afebrile and completely asymptomatic throughout the study. Leukopenia was present from Day 13 to 16.
2. WLO experienced mild pruritus and slight redness at the site of vaccine inoculation from Day 1 to 3, but did not develop regional lymph node enlargement. Throughout the first 10 days of study he experienced wide daily changes in temperature with peaks between 99 and 100°F. Following the onset of viremia on Day 9 his daily temperatures fluctuated at a slightly higher range and reached a maximum of 100.4F on Day 15. On Day 12 he complained of mild fatigue and photophobia. On Days 13 and 14 he was leukopenic.
3. GR had no fever, viremia or leukopenia. His only complaint was fatigue on Day 15 which he attributed to the resumption of duties after the period of isolation.
4. WHB remained afebrile throughout the study. Viremia was detected from Day 9 to 14. He complained of a bitemporal headache on Days 9 and 10. His total leukocyte count tended to be lower than the others throughout the study. On Day 7, the WBC count was 3600; this was the only low WBC count that occurred before the onset of viremia. Leukopenia returned from Day 15 to 21.
5. DDB was viremic from Day 9 to 13. On Day 12 he became febrile and complained of mild chills, headache and pain in the shoulders, knees and eyes. His temperature reached 99.8F on Day 12. A fine erythematous macular rash of the trunk and abdomen which extended into the axillae was observed from Day 12 to

17 (Figure 2). The rash faded completely with light pressure. Petechiae did not appear. Leukopenia was present from Day 14 to 17. He was not too ill to miss work despite the fever, symptoms and rash.

6. MAU had detectable viremia on Day 9 only. On the evening of Day 10 his temperature was 100F and reached 101.8 on Day 11. Medical consultants decided he was too ill to return to work. He reported fatigue on Day 11 followed by headache, muscle pain and photophobia. Rash and lymphodenopathy were not observed. Following the onset of fever and viremia his WBC count dropped to 4000 on Day 11, but not low enough to be considered leukopenia.

None of the volunteers complained of vomiting, diarrhea, abdominal pain, epistaxis or melena. No one developed petechiae, regional or generalized lymph node enlargement, abdominal tenderness, hepatomegaly, splenomegaly or conjunctional suffusion.

There was a tendency for a reduction in systolic and diastolic blood pressure during the 10 days of isolation but no narrowing of pulse pressure. Pulse rates rose during periods of fever. There were no changes or abnormal values in platelet counts, hemoglobin, hematocrit, serum biochemistries or serum complement values which could be attributed to the vaccine. Daily determinations of fibrin split products were uniformly negative. Urinalyses were normal throughout the study for all volunteers.

c. Antibody Response to Dengue 2 Vaccine

Antibody responses to immunization were measured by hemagglutination inhibition (HI), complement fixation (CF) and plaque reduction neutralization tests (PRNT). The prototoye strains of flaviviruses used for these tests, included dengue type 1, Hawaii; dengue type 2, New Guinea-C; dengue type 3, H-87; dengue type 4, H-241; Japanese encephalitis, M1/311; and yellow fever, 17-D. HI tests were done using 8 units of suckling mouse brain antigen; CF tests used 4 units of sucrose-acetone extracted antigens.

Plaque reduction neutralization tests were performed by mixing equal volumes of serum and virus to get a final

concentration of 30-100 pfu/0.1 ml. Virus-serum mixtures were incubated at 36°C for 30 minutes (vaccine virus, S-1 and vaccine parent virus, PR-159) and 60 minutes for the prototype dengue strains. Cells were washed and overlaid with agar. The plaques were counted after 7 days. Neutralizing antibody titers were determined by calculating the serum dilution that would give a 50% plaque reduction endpoint.

The five viremic volunteers developed four-fold or greater HI and PRNT antibody titers to DEN-2 (Figure 3). All but MAU also developed CF antibody. The broadly reactive HI antibody was typical of a recent dengue infection in people with heterotypic flavivirus immunity. In general, the highest DEN-2 antibody titers were found in the people with the greatest time interval since the last YF vaccination (Table 5).

Volunteer GR was found to have PRNT antibody on Day 0 to the vaccine parent strain PR-159 but not the prototype New Guinea-C. He showed the least antibody response to vaccination of any of the volunteers. There was no increase in neutralizing antibody to dengue virus and but minimal rises in HI and CF antibody; responses which might have resulted from exposure to a nonreplicating antigen in the presence of neutralizing antibody. The absence of symptoms and viremia combined with the poor antibody response indicate GR may not have been infected by the DEN-2 vaccine.

The PRNT antibody titers to the vaccine virus, S-1, and the parent strain, PR-159, were nearly identical (Table 6). A greater difference was found between the titers to PR-159 and the prototype New Guinea-C (NG-C). Detailed results of all laboratory tests for each volunteer are provided in Tables 7-21.

4. Discussion

This study demonstrated that an infectious dose of 10^5 virus particles infected all five susceptible recipients. Despite the appearance of viremia in five people and mild febrile illness in two people, no evidence was found of reversion of virus to virulence in vitro. The broadly reactive immune responses were expected in people with heterotypic flavivirus antibody. It is possible that equivalent antibody responses could have been obtained with fewer symptoms if a lower dosage had been used. The S-1 vaccine is sufficiently safe and immunogenic as to warrant testing of equal or lower dosages in additional human volunteers.

Table 5. Relationship of Time Interval Between Flavivirus Vaccines and DEN-2 Antibody Titer

Volunteer	Months Since YF Vaccine	DEN-2 Viremia	Dengue 2 Antibody Titer at 30 Days			
			CF NG-C	HI NG-C	PRNT NG-C	PRNT PR 159
MAU*	3	+	<8	80	49	60
FHT	21	+	64	640	70	140
DDB	48	+	64	160	83	160
GR	105	0	8	40	<10	20
WHB	115	+	256	2560	125	350
WLO*	196	+	512	2560	120	400

*Febrile volunteers

Table 6. PRNT Titers* of DEN-2 Vaccine Recipients

Vol.	Study Day	DEN-2			DEN-1	DEN-3	DEN-4
		Vaccine Virus (S-1)	Parent Virus (PR-159)	Prototype (NG-C)	Hawaii	(H-87)	(H-241)
FHT	0		<10	<10	<10	<10	<10
	30		140	70	66	16	86
	60	140	140				
WLO	0		<10	<10	<10	<10	<10
	30		400	120	220	60	220
	60	210	230				
GR	0		20	<10	33	<10	19
	30		20	<10	30	<10	20
	60	40	20				
WHB	0		<10	<10	<10	<10	<10
	30		350	125	250	75	270
	60	850	500				
DDB	0		<10	<10	<10	<10	<10
	30		160	83	170	40	140
	60	120	100				
MAU	0		<10	<10	<10	<10	<10
	30		60	49	33	10	30
	60	80	60				

*Reciprocal of the serum dilution resulting in 50% plaque reduction

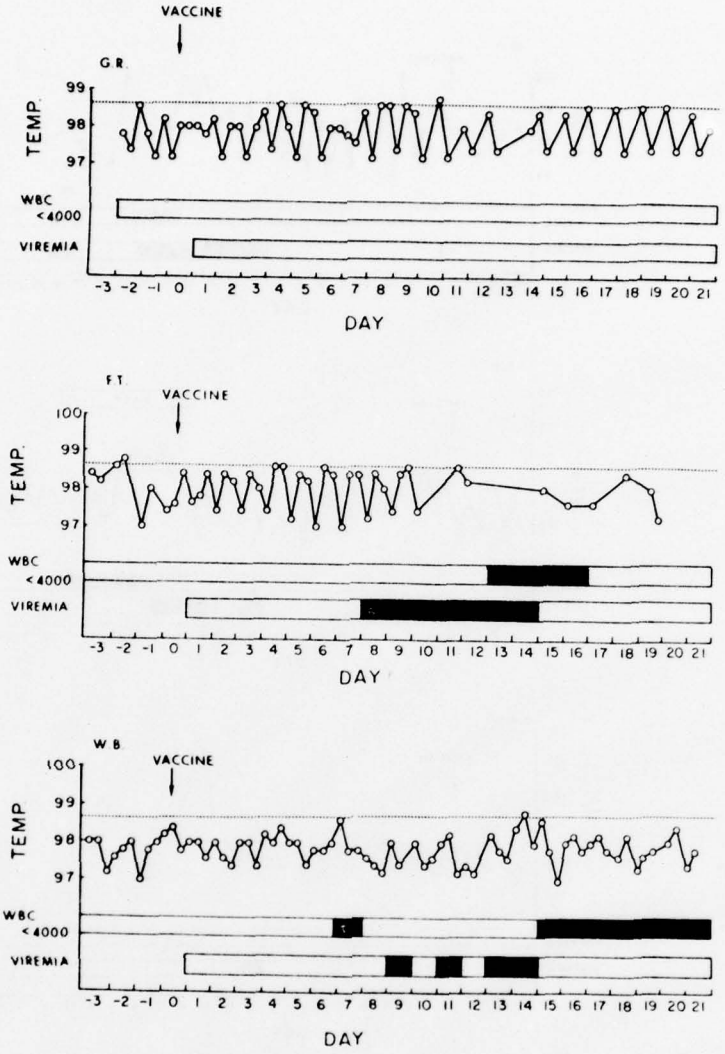


Fig. 1a. Relationship of temperature and total leukocyte (WBC) count to viremia in recipients of dengue 2 vaccine. Dotted line represents 98.6°F. Horizontal bars indicate the days tested. Shaded areas indicate the days of leukopenia (total WBC < 4000/mm^3) and virus isolation from plasma.

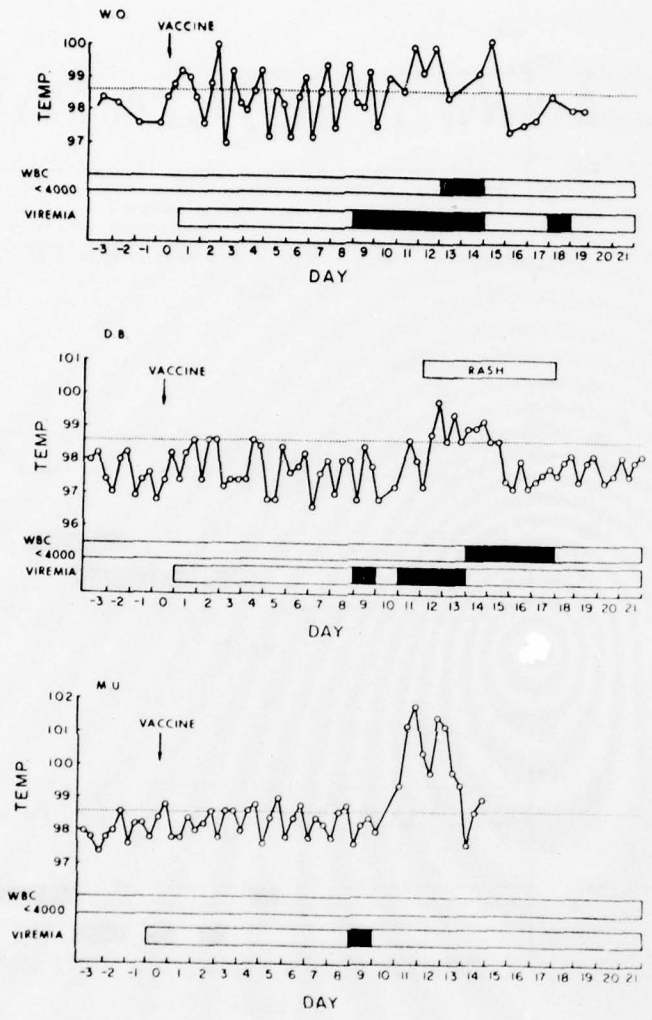

W O
VACCINE
TEMP
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99
98
97
WBC
< 4000
VIREMIA
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAY
D B
VACCINE
RASH
TEMP
101
100
99
98
97
96
WBC
< 4000
VIREMIA
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAY
M U
VACCINE
TEMP
102
101
100
99
98
97
WBC
< 4000
VIREMIA
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAY

Fig. 1b

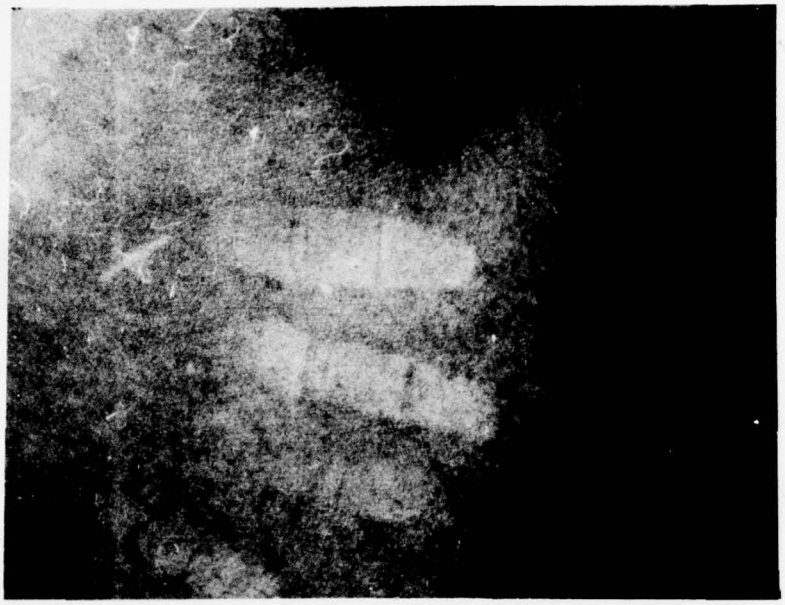


Fig. 2. Erythematous, mascular, nonhemorrhagic, non-pruritic rash of DDB which blanched completely on light pressure.

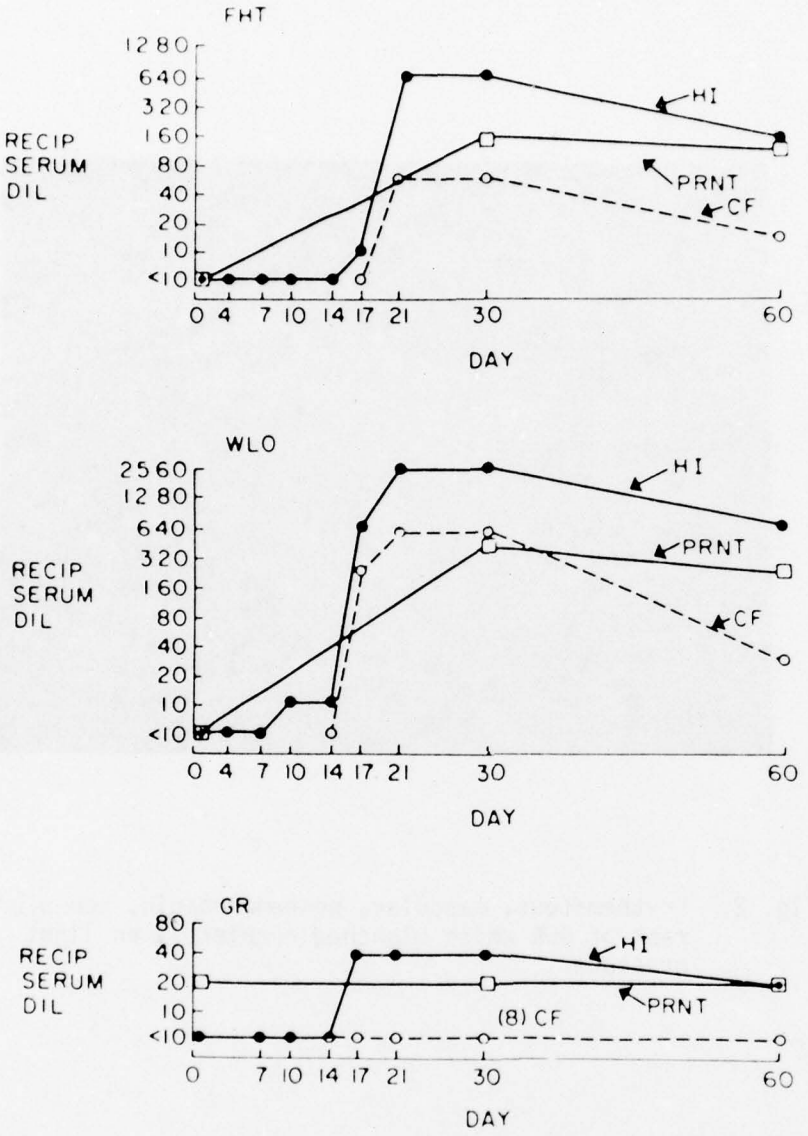


Fig. 3a. Dengue 2 antibody responses to a live dengue 2 vaccine given on Day 0. HI hemagglutination inhibition; CF is complement fixation; PRNT is plaque reduction neutralization test.

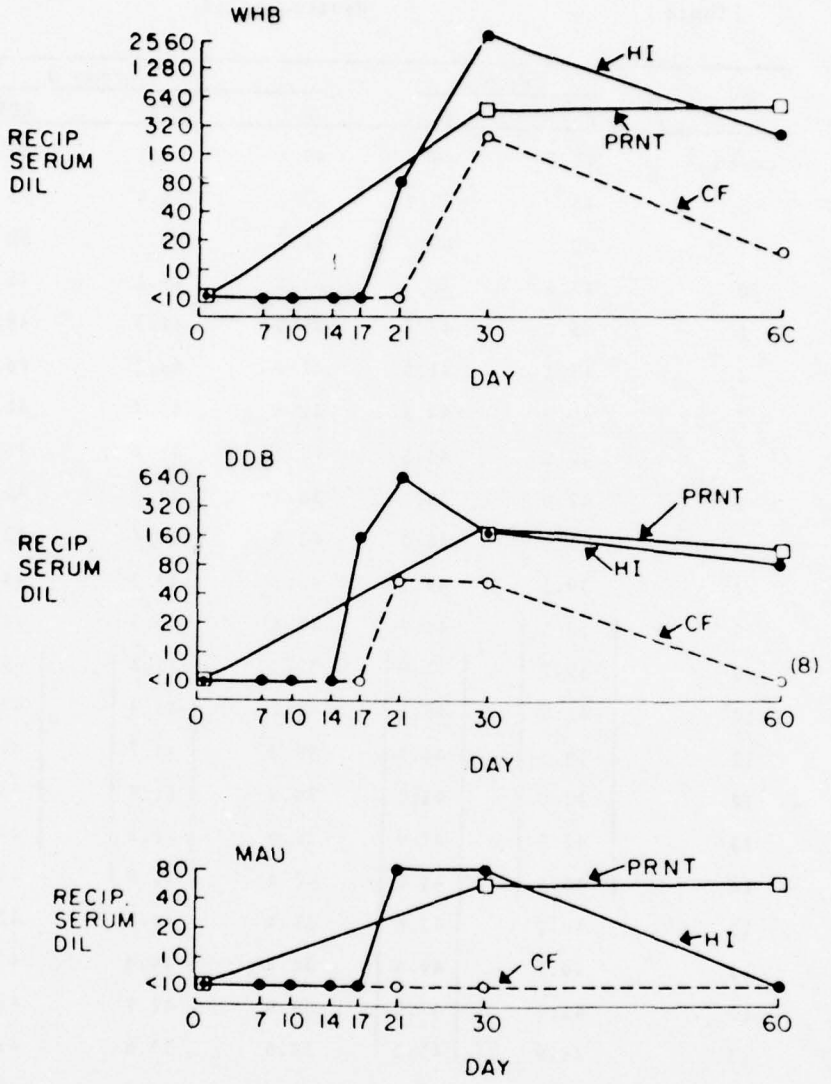

WHB
RECIP SERUM DIL
2560
1280
640
320
160
80
40
20
10
<10
HI
PRNT
CF
0 7 10 14 17 21 30 60
DAY
DDB
RECIP SERUM DIL
640
320
160
80
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20
10
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PRNT
HI
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0 7 10 14 17 21 30 60
DAY
MAU
RECIP. SERUM DIL
80
40
20
10
<10
PRNT
HI
CF
0 7 10 14 17 21 30 60
DAY

Fig. 3b

Table 7 Hematocrit (%)

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	41.7	48.6	43.1	43.1	44.0	46.5
-3	43.5	50.6	ND*	48.4	45.6	41.6
-2	ND	ND	41.9	ND	ND	ND
0	43.6	48.2	44.2	45.1	45.4	42.3
1	43.0	47.8	45.0	44.3	45.9	41.8
2	43.1	41.9	41.9	48.1	46.9	44.3
3	40.5	44.8	42.8	45.3	46.3	43.1
4	40.1	44.5	42.1	45.2	45.0	42.8
5	42.0	46.0	38.3	42.3	40.4	40.1
6	43.0	46.0	42.6	44.4	42.9	42.4
7	39.1	44.2	40.5	43.2	44.5	43.4
8	38.5	45.9	42.6	45.3	44.1	40.1
9	39.7	42.5	39.1	42.3	40.1	39.6
10	41.1	46.1	41.2	45.9	42.5	39.8
11	39.3	44.5	39.2	44.7	40.4	41.5
12	42.0	43.2	39.6	51.8	43.1	44.4
13	41.0	42.9	39.6	43.2	44.3	43.5
14	43.5	45.9	43.9	45.8	43.9	41.3
15	40.6	43.6	39.4	44.4	42.8	41.8
16	40.7	46.8	36.8	44.8	40.4	38.7
17	41.3	47.0	39.9	42.9	41.6	39.2
18	38.9	45.2	38.6	37.8	41.7	40.4
19	41.2	42.4	43.8	46.0	46.0	40.0
20	40.9	42.8	40.9	46.4	41.4	39.4
21	ND	42.0	39.9	43.0	40.3	42.5

* Not determined

Table 8 Total WBC Count (X1000)*

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	5.0	8.2	7.5	5.0	5.3	6.7
-3	5.3	8.0	ND**	4.7	5.7	5.6
-2	ND	ND	6.4	ND	ND	ND
0	6.5	7.8	6.7	5.0	5.3	5.2
1	11.5	9.7	5.5	4.7	5.8	5.4
2	6.0	7.2	5.7	5.2	6.7	6.1
3	5.6	7.3	5.5	5.9	5.1	5.3
4	5.8	6.9	5.8	5.1	4.9	6.8
5	6.1	7.9	5.9	4.7	4.8	6.9
6	6.5	7.3	7.1	4.0	4.8	7.4
7	5.9	7.8	5.8	3.6	4.9	6.2
8	5.7	7.7	6.3	4.0	5.0	6.6
9	5.2	7.1	6.7	4.6	5.1	6.4
10	5.9	8.0	6.4	4.2	5.2	5.3
11	4.4	6.4	6.3	4.6	4.2	4.0
12	6.0	7.3	6.1	4.6	4.0	4.8
13	3.2	3.6	6.5	4.5	4.3	6.3
14	3.7	3.8	6.1	4.3	3.9	5.4
15	3.6	4.1	5.3	3.4	3.2	5.0
16	3.6	4.3	5.2	2.8	3.4	6.0
17	4.0	5.0	5.3	2.9	3.2	5.8
18	4.7	5.8	5.7	3.1	4.1	6.3
19	5.1	6.4	6.0	3.5	4.7	6.7
20	5.6	6.7	5.9	3.2	4.3	6.2
21	ND	6.5	6.8	3.9	4.7	6.1

*WBC below 4000 considered leukopenia.

**Not determined

Table 9 Platelet Counts (X1000)

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	330	365	250	245	275	240
-3	285	250	ND*	350	280	300
-2	ND	ND	260	ND	ND	ND
0	215	305	275	275	305	285
1	295	370	185	375	205	235
2	295	325	205	220	225	210
3	590	565	195	170	180	260
4	265	320	205	260	195	250
5	260	310	265	170	170	340
6	275	325	150	240	275	235
7	270	345	245	250	280	270
8	265	310	205	285	300	300
9	260	375	180	255	295	225
10	330	395	265	255	230	170
11	280	300	220	270	155	190
12	285	360	180	260	185	245
13	300	355	175	225	175	215
14	325	260	200	295	220	285
15	360	315	250	215	250	305
16	310	340	190	240	285	410
17	ND	325	175	270	190	345
18	355	240	ND	315	210	380
19	335	280	185	190	175	283
20	340	330	175	155	255	280
21	ND	335	230	265	280	395

* Not determined

Table 10 Serum C 3

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	166	194	ND*	ND	ND	ND
-3	178	173	ND	112	126	131
-2	ND	ND	170	ND	ND	ND
0	155	180	167	108	133	142
1	176	176	145	104	119	124
2	137	205	151	119	122	138
3	155	216	149	108	130	127
4	184	187	158	140	130	146
5	155	169	153	126	126	144
6	180	176	156	133	133	133
7	162	176	153	133	133	131
8	180	187	169	144	126	137
9	148	140	160	162	119	137
10	180	187	153	126	119	139
11	230	180	142	137	119	175
12	187	158	151	144	126	158
13	166	155	145	126	130	174
14	162	166	131	119	119	140
15	155	166	138	122	130	150
16	155	212	130	122	115	150
17	176	202	143	126	115	131
18	144	173	133	119	108	124
19	169	176	142	133	101	127
20	155	176	132	108	97	113
21	119	176	138	119	97	108

* Not determined

Table 11 Serum SGOT*

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	10.0	12.0	10.0	15.0	10.0	3.0
0	4.3	5.0	12.0	12.0	9.0	8.0
2	7.2	9.6	5.2	6.2	0.9	6.2
4	4.0	7.0	8.6	12.0	8.6	10.0
6	3.8	8.2	11.0	8.1	13.9	8.6
8	8.0	14.0	5.2	6.2	7.6	6.2
10	8.0	9.0	15.8	15.8	20.6	11.0
12	7.0	10.0	11.0	12.0	24.4	23.0
14	4.0	8.0	10.0	28.0	7.2	16.8
16	6.0	7.0	6.7	10.0	3.8	10.0
18	4.0	6.0	10.0	8.6	3.8	6.7
20	5.0	6.0	10.0	10.0	7.0	8.0

* Normal values: 0-13 Iu/L

Table 12 Serum SGPT*

Day	GROUP A		GROUP B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	10.0	13.0	10.0	26.0	3.0	1.0
0	1.2	1.9	11.0	15.0	8.0	9.0
2	14.0	9.6	9.6	5.2	6.2	5.2
4	5.0	4.8	17.7	10.0	17.7	11.0
6	4.8	7.2	12.0	6.2	9.1	10.0
8	7.0	9.0	4.3	7.2	8.1	6.2
10	8.0	7.0	13.4	12.0	16.8	11.0
12	8.0	7.0	9.1	11.0	13.4	18.7
14	5.0	6.0	18.2	9.1	7.2	16.3
16	3.0	8.0	11.0	12.0	0.9	6.2
18	6.0	7.0	11.0	13.4	0.5	6.2
20	4.0	8.0	11.0	10.0	7.0	6.0

* Normal values: 0-10 IU/L

Table 13 Serum LDH*

Day	Group A			Group B		
	FHT	WLO	GR	WHB	DDB	MAU
Screen	86	77	58	67	60	80
0	125	115	77	77	77	54
2	175	168	70	260	70	160
4	129	125	38	53	38	48
6	125	120	19	34	53	62
8	125	134	96	77	49	120
10	106	106	46	35	43	43
12	118	113	105	134	125	76
14	119	126	192	182	168	105
16	100	115	96	67	67	53
18	110	125	115	ND**	ND	ND
20	112	120	96	57	67	77

* Normal values: 48-168 IU/L

** Not determined

Table 14 Blood Urea Nitrogen*

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	19	14	16	12	12	15
0	16	11	18	13	17	15
2	18	15	18	18	17	13
4	16	10	16	17	16	8
6	16	11	13	14	17	10
8	9	13	20	16	23	12
10	17	12	15	15	12	12
12	15	13	16	18	11	14
14	20	11	16	16	26	11
16	19	12	8	21	26	9
18	18	11	16	21	25	21
20	17	9	16	14	15	13

* Normal values: 9-20 mg/dl

Table 15 Serum Creatinine*

Day	Group A		Group B			
	FHT	WLO	GR	WHB	DDB	MAU
Screen	ND**	ND	1.4	ND	0.8	0.7
0	1.3	1.4	1.0	1.6	1.0	1.0
2	0.8	1.0	1.5	4.0	1.1	1.1
4	1.3	0.8	1.3	1.2	1.0	1.3
6	1.4	0.9	1.0	1.2	0.8	0.8
8	0.8	1.1	0.7	0.8	0.9	0.8
10	1.2	0.9	1.4	1.3	1.3	1.1
12	1.3	0.9	1.3	1.3	1.3	1.3
14	1.3	0.9	1.3	1.4	1.3	1.4
16	1.3	0.8	1.3	1.5	1.1	1.2
18	1.2	0.8	1.2	1.4	1.3	1.3
20	1.3	0.9	1.2	1.0	1.2	1.1

* Normal values: 0.7-1.4 mg/dl

** Not determined

Table 16. HI and CF* Antibody Responses of Volunteer F.H.T.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	<10	<10	<10	<10
4	<10	<10	<10	<10	<10	<10
7	<10	<10	<10	<10	<10	<10
10	<10	<10	<10	<10	<10	<10
14	<10	<10 (<8)	<10 (<8)	<10	<10	<10
17	<10	10 (<8)	20 (<8)	10	20	20
21	160	640 (64)	640 (32)	1280	1280	2560
30	320	640 (64)	640 (32)	2560	1280	5120
60	80	160 (16)	80	640	1280	160

*CF titers enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

Table 17. HI and CF* Antibody Responses of Volunteer W.L.O.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	<10	<10	10	40
4	<10	<10	<10	<10	10	40
7	<10	<10	<10	<10	10	40
10	<10	10	<10	<10	10	40
14	<10	10 (<8)	<10 (<8)	10	20	80
17	640	640 (256)	2560 (256)	10240	20480	10240
21	2560	2560 (512)	5120 (256)	10240	20480	20480
30	1280	2560 (512)	2560 (256)	10240	20480	20480
60	320	640 (32)	160	2560	640	640

*CF titers enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

Table 18. HI and CF* Antibody Responses of Volunteer G.R.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	20	40	40	80
7	<10	<10	<10	40	40	40
10	40	<10	20	80	40	80
14	40	<10 (<8)	20	20	160	160
17	40	40 (<8)	20	80	160	80
21	80	40 (<8)	20	80	160	80
30	40	40 (8)	20	160	160	80
60		20 (<4)				

*CF titers are enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

Table 19. HI and CF* Antibody Responses of Volunteer W.H.B.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	<10	<10	<10	<10
7	<10	<10	<10	<10	<10	<10
10	<10	<10	<10	<10	<10	<10
14	<10	<10 (<8)	<10	<10	<10	<10
17	40	<10 (<8)	<10	<10	<10	<10
21	80	80 (<8)	80	160	640	160
30	640	2560 (256)	2560	5120	5120	2560
60		320 (16)				

*CF titers enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

Table 20. HI and CF* Antibody Responses of Volunteer D.D.B.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	<10	<10	<10	<10
7	<10	<10	<10	<10	<10	<10
10	<10	<10	<10	<10	<10	<10
14	<10	<10 (<8)	<10	<10	<10	<10
17	<10	160 (<8)	160	640	320	320
21	320	640 (64)	320	5120	20480	2560
30	160	160 (64)	640	2560	1280	640
60		80 (8)				

*CF titers enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

Table 21. HI and CF* Antibody Responses of Volunteer M.A.U.

DAY	Mouse Brain Antigens**					
	DEN-1	DEN-2	DEN-3	DEN-4	JE	YF
0	<10***	<10	<10	<10	<10	<10
7	<10	<10	<10	<10	<10	<10
10	<10	<10	<10	<10	<10	<10
14	<10	<10 (<8)	<10	<10	<10	20
17	<10	<10 (<8)	<10	20	<10	20
21	40	80 (<8)	40	320	160	160
30	80	80 (<8)	160	640	160	160
60		<10 (<4)				

*CF titers enclosed in parentheses.
**Antigens were prepared to prototype viruses.
***Reciprocal serum dilution

B. Effect of immunization with live dengue type 2 vaccine virus on yellow fever neutralization antibody titers.

Yellow fever plaque reduction neutralization tests were carried out to determine the quantitative effects of a live DEN-2 vaccine virus on antibodies specific for the serologically related yellow fever virus. All individuals had been immunized previously with live yellow fever vaccine virus. The HI test results, presented in the previous section, showed a considerable rise in titer to the flavivirus antigens that were used (JE, YF, and DEN), and point out that the HI test is non-specific and measures flavivirus group-reactive antibodies. While two individuals had pre-existing YF HI antibodies (Tables 17,18), all six had pre-existing yellow fever neutralizing antibodies (Table 22); essentially no change in titer of yellow fever PRNT antibodies was observed by Day 30, a period of time where dengue PRNT antibodies had risen considerably (compare Table 5 to Table 22).

Table 22. Yellow Fever Neutralization (PRNT) and Hemagglutination-Inhibition (HI) Titers of Sera from 6 Volunteers Immunized with Live DEN-2 Vaccine

Volunteer	Months since YF Immunization	YF PRNT		YF HI	
		Day 0	Day 30	Day 0	Day 30
MAU	3	160	320	<10	160
FHT	21	72	90	<10	5120
DDB	48	22	36	<10	640
GR	105	80	70	80	80
WHB	115	23	40	<10	2560
WLO	196	70	90	40	20480

The individual with the highest YF neutralizing antibody titer was the most recent recipient of yellow fever vaccine (Table 22) and the only individual to become ill following immunization with the dengue vaccine. Temporal relationships between time of exposure to various flaviviruses and severity of disease are not yet understood. One possible mechanism is "infection enhancement" with critical levels of closely related heterotypic antibody as described by Halstead and described in the previous annual report.

C. Qualitative differences in the complement fixing (CF) antibody response to the dengue 2 live virus vaccine and to wild dengue infections.

The CF antibody responses in clinical dengue cases are measured in this laboratory with high-speed supernatant fluids of 20% infected mouse brain suspensions. CF titers of sera from primary dengue cases are as high as 1:128; and as high as 1:2048 in sera from secondary cases of dengue (previous annual reports). The sera from the volunteers infected with the attenuated dengue 2 vaccine virus did not produce CF antibodies reactive with the crude antigen. The sera were tested again with a solvent extracted antigen prepared by the sucrose acetone method of Clarke and Casals (1958). The CF titers obtained with this antigen were presented in a previous section of this report, and were as high as 1:512. The sucrose acetone antigen is rich in virion structural components (hemagglutinins) while the crude antigen contains all of the virus specified products, both structural and nonstructural antigens. Thus, individuals infected with the vaccine virus may have produced antibodies to the virion structural antigens whereas individuals exhibiting clinical symptoms from infection with a wild virus produce antibodies to both structural and nonstructural antigens. Lysis of infected cells in classical dengue may be the source of the increased number of viral immunogens to which the host produces antibodies

D. Effect of dengue-2 virus passage history on the susceptibility of human monocytes to productive infection in vitro and ability of monocytes to support replication after infection in vivo.

1. Background

The replication in human monocytes of dengue-2 virus and an attenuated live virus vaccine candidate derived from it, was described in the previous annual report. Briefly, the two viruses which appear to be identical antigenically, can be differentiated easily from one another biologically: 1. the parent virus produces a mixture of large and small plaques on LLC-MK_2 monolayers, while the vaccine candidate produces only small plaques and is, therefore, designated as a small plaque (SP) clone; 2. the parent virus replicated at 39 C while the SP clone is restricted at this temperature; 3. the parent virus produces viremia in primates while the SP clone produces little

if any viremia; 4. the parent virus at low concentrations is lethal for suckling mice by the intracerebral route while the SP clone is not lethal; 5. in human monocytes the parent virus replicated rapidly to high titer while the SP clone replicated to a low titer in a delayed manner or it did not replicate at all.

The viruses were propagated in primary African green monkey kidney (GMK) cells certified for the production of vaccines. However, a position was developed by the Bureau of Biologics that a product from GMK cells, while appropriate for oral administration (i.e., live attenuated polio virus), was not appropriate for parenteral administration due to the possible inoculation of unknown carrier agents into susceptible tissue. A diploid cell line derived from fetal rhesus lung (FRhL) was characterized and certified by Bureau scientists as acceptable for production of a live attenuated product for parenteral inoculation.

Passage of the GMK propagated viruses into FRhL cells was described in the previous Annual Report. It was found that the FRhL cell propagated SP clone retained the first 4 characteristics listed above (produced small plaques, was temperature sensitive, did not produce viremia in sub-human primates, or kill suckling mice). During the past year, we found that the FRhL propagated SP virus regained the ability to replicate in human monocytes in vitro in the same manner as the wild virus. However, when the SP clone was inoculated into human volunteers, it did not infect monocytes in vivo as did the wild virus, a result predicted with the GMK passaged SP virus, but not predictable with the FRhL passaged SP virus.

2. Methods

Prior to inoculation with the live dengue type 2 virus vaccine in Study No. 1, 10 ml of blood was taken from each of the 6 human volunteers with a commercial vacuum tube containing 143 units of preservative-free dried heparin (green top vacutainer). The blood was centrifuged at 750 x g for 10 minutes at 4 C and the plasma was removed immediately (4 ml) and replaced with the same volume of Puck's saline containing 0.0025 M EDTA and 0.01 M Hepes buffer. The tubes were transported from USAMRIID to WRAIR where the 10 ml blood mixture was diluted to 25 ml in a 50 ml plastic conical centrifuge tube with Ca and Mg free Hanks' balanced salt solution containing EDTA and Hepes buffer; 15 ml of ficoll hypaque (sp. gr. 1.077) was

injected underneath each sample and the tubes were centrifuged at 350 x g for 40 minutes at room temperature. The white cell band was collected, washed, and separated into adherent and nonadherent cells as described in the previous annual report. The adherent cells (monocytes or mononuclear phagocytes) in 25 cm^2 flasks were infected with an aliquot of the live virus vaccine as well as the parent virus from which it was derived. The inoculum was removed and residual virus was decreased to the indicated background levels by rinsing the growth surface of the culture flask several times. Six ml of growth medium (RPMI 1640 containing 10% fetal bovine serum, Hepes buffer and antibiotics) was placed in the flasks and 1 ml was removed immediately for a 0 time sample. Daily samples of 1 ml each were removed and replaced with fresh medium for 5 days. Virus produced by the monocytes was measured by plaque assay in LLC-MK_2 cells.

After the volunteers were inoculated with the live virus vaccine, 10 ml of blood was drawn as above for 21 days. The plasma was saved for virus isolation and the adherent cells were assayed by the infectious center method to determine the presence of infected monocytes. Between 1.8 to 6.3 x 10^5 adherent cells were overlaid with sufficient LLC-MK_2 cells to form a confluent monolayer in 24 hours; the following morning they received an agar overlay. Another method used to detect infected monocytes from the volunteers was to culture the adherent cells for 5 days and remove samples of supernatant fluid daily. These samples were tested for infectious virus by plaque assay on LLC-MK_2 cells. Based on in vitro studies and results from the first two volunteers, monocyte cultures from the second group were tested for infectious virus only after 5 days of culture.

Nonadherent cells (lymphocytes) from the second group of volunteers were also tested for virus. The lymphocytes were cultured for 5 days at which time the mixture of suspended cells and media was frozen at -70 C until tested for virus by plaque assay.

3. Results

The replication of the parent D-2 virus and the FRhL SP clone vaccine was determined in the preimmunization monocytes from 3 volunteers (FHT, WLO, and WHB). The vaccine virus replicated to as high if not higher titer than the parent virus (Figure 4).

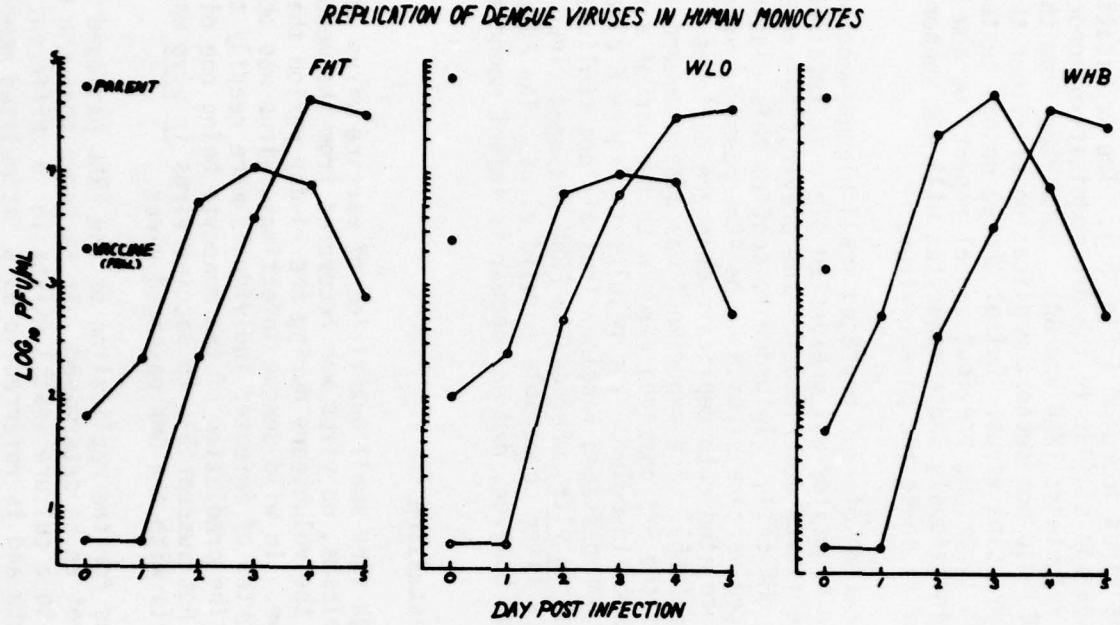


Fig. 4. In vitro infection of pre-immunization human monocytes with the dengue-2 parent and vaccine viruses.

The 1-day delay in vaccine virus replication was probably due to the lower titer of vaccine virus inoculum. This reasoning was supported in the next experiment where the parent virus inoculum was diluted down to the same potency as the vaccine virus inoculum (Fig. 5). The replication of both viruses was similar in the preimmunization monocytes from 2 other volunteers (MAU and GR). Monocytes from the 6th volunteer (DDB) did not support replication of either the parent or the vaccine virus. Out of the 23 donors tested up to the present time (see previous annual report) we now have found only 2 individuals whose monocytes will not support replication of at least the parent virus.

The replication of FRhL passaged small plaque vaccine virus in human monocytes was unexpected since it retained the other characteristics described for the previous passages of this virus in GMK cells. In order to confirm this distinction, the GMK passaged vaccine virus and the FRhL passaged vaccine virus were inoculated onto replicate monocyte cultures from the same donor (WHB). The experiment was biased toward the replication of the GMK passaged virus with the use of a slightly higher titered inoculum. The results in Figure 6 clearly show that the GMK passaged vaccine virus did not replicate in human monocytes in vitro whereas the FRhL passaged virus again replicated to a titer comparable to wild virus. The FRhL passaged virus, however, did not appear to infect monocytes in vivo.

4. Discussion

Even though very small quantities of vaccine virus was found in the plasma, no virus was recovered from the monocytes cultured from the volunteers during the 21-day period that blood was drawn. In wild dengue infections, virus was obtained from the monocytes of infected individuals more readily than from plasma. The prediction of the monocyte being one of the cells that is not susceptible to vaccine virus in vivo was based on results with the GMK passaged virus.

The reasons for the replication of the FRhL passaged virus in vitro has yet to be determined. It is known that the monocyte attached to a culture vessel exists in a different physiological state and is referred to as a "stimulated monocyte." The susceptibility of monocytes in vitro to parent and FRhL vaccine virus will be tested by infecting them in suspension and then sampling them from suspension cultures as well as adherent cultures prepared post-infection.

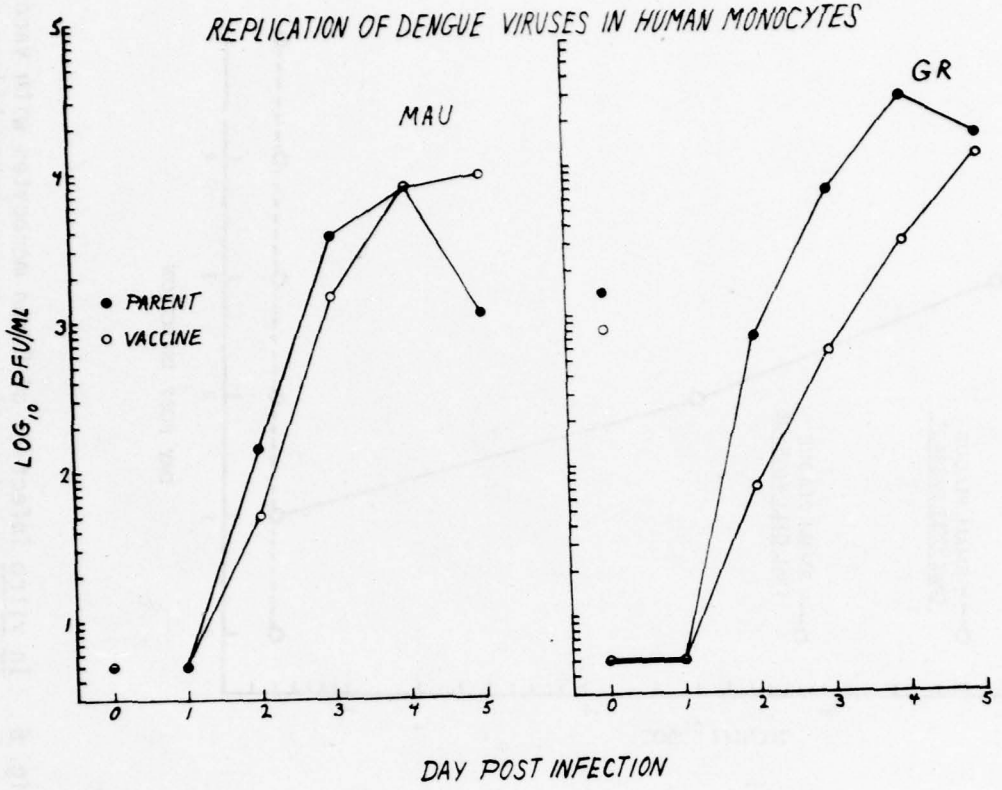


Fig. 5 . In vitro infection of pre-immunization human monocytes with the dengue-2 parent and vaccine viruses at similar multiplicities of infection.

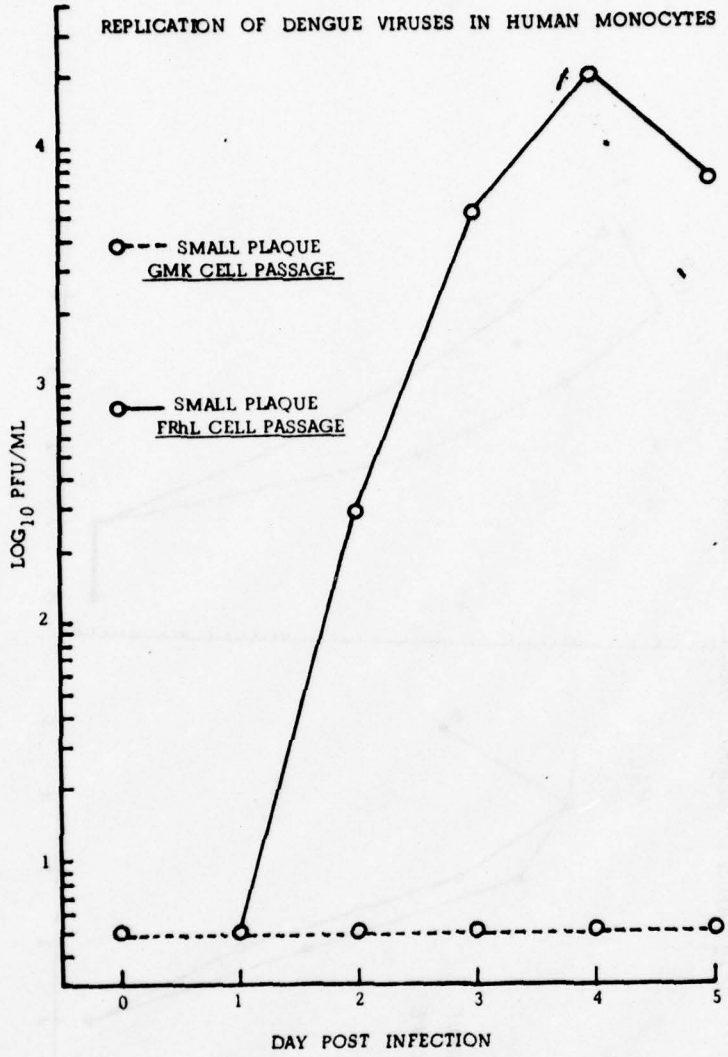


Fig. 6. In vitro infection of human monocytes with vaccine virus passaged in African green monkey kidney cells.

E. Identification of Dengue Viruses from the Caribbean by Plaque Reduction Neutralization Test.

1. Background

The introduction of dengue type 1 virus to the Caribbean in 1977 caused widespread disease in a near totally susceptible population. Since dengue types 2 and 3 were already present in the region, description of the epidemiology of dengue type 1 disease required a reliable and precise means for differentiating viral serotypes. A combination of virus amplification in cell culture, plaque assay and plaque reduction neutralization test provided a useful procedure for virus isolation and a highly reliable and specific method for serotype identification.

2. Methods

a. Sources of Viruses

Acute serum specimens were collected by PAHO-sponsored field teams in Jamaica and the Bahamas or referred by investigators in Cuba and Puerto Rico. Viruses isolated in Aedes pseudoscuttellaris cell cultures were submitted in the form of infected cell culture fluids by the Caribbean Epidemiologic Center in Trinidad. Viruses isolated by inoculation of Aedes aegypti were submitted as pools of whole mosquitoes by the San Juan Laboratory in Puerto Rico. All specimens were originally collected between 10 July 1977 and 19 January 1978.

b. Virus Amplification by Passage in LLC-MK_2 Cells

Each diagnostic sample was carried through at least one passage in LLC-MK_2 cells under liquid medium in order to amplify the virus titer. In this step, 0.2 ml of the original sample was allowed to adsorb to a confluent cell monolayer at 35°C for one to two hours. To this was added 5.0 ml of medium 199 containing 2% fetal bovine serum, 100 units/ml penicillin and 100 mcg/ml of streptomycin. Seven days post inoculation, the medium was removed and stored at -70°C until it was tested by plaque assay. Fresh medium was placed on the monolayer until 14 days post-inoculation at which time the medium was removed, the cells were frozen and thawed, and then suspended in the same medium. Samples harvested in this manner were centrifuged at 350 x g for 10 min and stored at -70°C until tested for virus by plaque assay. If no virus was detected, the amplification

step under liquid medium was repeated by inoculating the 14-day cell culture harvest from the previous passage. Specimens were considered to be negative only when no virus was detected after three successive passages under liquid medium.

c. Plaque Assay

For the plaque assay (Eckels et al., 1976), serial 10-fold dilutions were made of the sample to be tested and 0.2 ml of each dilution was allowed to adsorb to the cell monolayers for one hour at 35°C. Each monolayer was then overlaid with 7.0 ml of an enriched medium containing 1% purified agar at 44°C (Table 23). After incubation for 6 days at 35°C, the flasks were

Table 23. Preparation of First Overlay Medium

A.	Agar Portion	
1.	Difco agar purified	1.0 gm
2.	Double distilled water	74 ml
B.	Nutrient Protion	
1.	Medium 199 (10 X)	10 ml
2.	Fetal bovine serum*	10 ml
3.	Sodium bicarbonate (7.5%)	4 ml
4.	Diethyl aminoetyl-dextran (2.0%) in Hank's balanced salt solution	1.0 ml
5.	Eagle basal medium vitamins (100 X)	0.5 ml
6.	Eagle basal medium amino acids (100 X)	0.5 ml
C.	Preparation	
1.	Sterilize agar portion in autoclave.	
2.	Mix ingredients of nutrient portion in order listed to avoid the formation of precipitates.	
3.	Combine agar portion with nutrient portion at 44°C.	

*Fetal bovine serum must be previously determined to be non-toxic for LLC-MK_2 cells at a concentration of 20%.

overlaid a second time with 4.0 ml of normal saline containing 1% agar and 1:1500 neutral red strain. Within 24 hours, plaques were counted over a transmitted light source. The quantity of virus was calculated as the number of plaque forming units (pfu) per milliliter.

d. Plaque Reduction Neutralization Tests

Dengue virus identification was accomplished with a standard plaque reduction neutralization test (PRNT) of Russell and McCown (1972) using virus grown under liquid medium which had been quantified by plaque assay. Virus identification was performed with hyperimmune mouse ascitic fluids prepared by standard techniques (Brandt et al., 1967; Chiewsilp and McCown, 1972), to reference strains of virus. Antibody titers in the typing fluids are shown in Table 24.

Table 24. Specificity of Hyperimmune Mouse Ascitic Fluids

		Mouse Ascitic Fluid			
		DEN-1	DEN-2	DEN-3	DEN-4
Dengue Type	Virus Strain	Hawaii	NG-C	PR-6	H-241
1	Hawaii	1500*	12	44	<10
2	New Guinea-C	30	1700	10	<10
3	Puerto Rico 6	10	10	>640	<10
4	Philippines H-241	10	10	20	160

*Reciprocal dilution giving 50% plaque reduction.

Two-fold dilutions of hyperimmune mouse ascitic fluids were mixed with equal volumes of virus suspension to give a final concentration of 30-100 pfu/0.1 ml and allowed to incubate in glass tubes for 30 min at 22°C. The virus-antibody mixture (0.2 ml) was allowed to adsorb to cell monolayers for one hour at 35°C, then was overlaid with agar as described above. Plaques were counted on day 7 and 50% plaque reduction endpoints were calculated by the method of Cutchins et al., (1960).

3. Results

One hundred seven (107) specimens from residents of nine countries were received for virus isolation and identification. From these specimens 53 strains of dengue virus were identified including 33 type 1, six type 2 and 14 type 3 viruses (Table 25). Dengue type 1 was found in all but one location sampled and was the only serotype identified from the Bahamas, Cuba, Guyana, Jamaica, St. Maarten and Trinidad and Tobago. Dengue types 1 and 2 were identified on Dominica; type 3 virus was found in the Dominican Republic. Only Puerto Rico submitted specimens which yielded all three dengue serotypes.

Of the 53 dengue strains isolated, 44 (84%) were detected by plaque assay of the original material submitted (Table 26). Preliminary passage of serum specimens in LLC-MK_2 cells under liquid medium increased the number of virus isolates from serum specimens from 23 to 31, but provided only one additional isolate from mosquito cell culture fluids and whole mosquitoes.

The plaque morphology was usually similar for viruses of the same serotype, although each type exhibited some degree of heterogeneity. Dengue types 1 and 3 usually produced small (<1-2 mm) clear plaques with less variation in size than was seen with type 2 strains (1-4 mm). Three strains of dengue type 1 detected in mosquito cell cultures were exceptional because they produced mixtures of small and very large (6-8 mm) plaques; such marked heterogeneity has not been previously observed in this laboratory (Figure 7). Whether or not this extreme plaque variation was present in the original serum or developed during passage through the mosquito cell culture is under investigation.

4. Discussion

Epidemiological studies of dengue require precise identification of virus isolates. In regions where two or more dengue serotypes coexist, a combination of plaque assay and plaque reduction neutralization tests in LLC-MK_2 cells has been used successfully to isolate and identify dengue viruses. The major advantages of these tests are a high degree of specificity and reliability plus the capability of detecting variant strains of virus based on differences in plaque morphology, temperature sensitivity or other growth characteristics (Eckels et al., 1976). In addition, the plaque reduction neutralization test can distinguish subtle antigenic differences of epidemiologic

Table 25. Identification of 1977 Caribbean Dengue Viruses

Origin of Strain	Virus Strain	Specimen*	Immune Ascitic Fluids			
			DEN-1	DEN-2	DEN-3	DEN-4
Bahamas	5 KS	S	220**	<10	10	<10
	8 HM	S	>320	12	10	<10
	9 SS	S	100	<10	<10	<10
	11 KJ	S	170	<10	11	<10
	12 AF	S	210	<10	10	<10
	13 AS	S	250	12	11	<10
	14 KC	S	230	19	23	<10
	15 NC	S	110	10	<10	<10
	19 VG	S	200	<10	<10	<10
	20 EL	S	80	<10	<10	<10
	23 VS	S	180	<10	14	<10
	24 JT	S	160	<10	<10	<10
	777849	C	175	11	<10	<10
	777882	C	160	<10	11	<10
	777898	C	135	<10	<10	<10
Cuba	1	S	200	<10	10	<10
	2	S	110	<10	<10	<10
	3	S	220	<10	<10	<10
	4	S	320	<10	32	10
Dominica	775270	S	115	<10	<10	<10
	775283	C	27	>80	22	<10
	775508	S	29	>80	16	10
Dominican Republic	14307	S	14	12	>160	<10
Guyana	778074	C	100	<10	< 10	<10
	778976	C	91	<10	< 10	<10
	779016	C	170	<10	< 10	<10
Jamaica	13802	S	70	<10	10	<10
	13806	S	150	<10	< 10	<10
	1515	S	88	<10	< 10	<10
	1536	S	100	<10	< 10	<10
	1570	S	100	<10	< 10	<10

*Specimens included serum (S), mosquito cell culture fluid (C) and inoculated whole mosquitoes (M).
**Reciprocal of 50% plaque reduction endpoint.

Table 25. (cont'd)

Country	Virus Strain	Specimen	Immune Ascitic Fluids DEN-1	DEN-2	DEN-3	DEN-4
Puerto Rico	23334	C	100	<10	10	<10
	23723	C	150	<10	<10	<10
	14236	S	<10	170	<10	<10
	14990	S	13	>80	11	<10
	90551	M	<10	>80	<10	<10
	93268	M	<10	>80	<10	<10
	14240	M	28	22	>320	<10
	14242	M	14	24	>320	<10
	14243	M	18	22	>320	<10
	14378	S	<10	<10	>160	<10
	14393	S	23	15	>160	<10
	15092	S	10	<10	>320	<10
	15353	S	16	12	>160	<10
	15354	S	10	12	>160	<10
	16985	M	<10	<10	>160	<10
	20162	M	<10	<10	>160	<10
	22574	M	<10	<10	>160	<10
	73026	M	<10	<10	>160	<10
	91150	M	<10	15	>160	<10
St. Maarten	22472	M	82	<10	< 10	<10
Trinidad and Tobago	780355	C	95	<10	< 10	<10
		C	170	<10	< 10	<10

Table 26. Detection of Dengue Virus by Plaque Assay

$LLC\text{-}MK_2$ Passage Level	Type of Specimen			
	Serum	Cell Culture	Mosquito	Combined
Original material	23	10	11	44
Passage 1 or 2	8	1	0	9
Total	31	11	11	53



Fig. 7. Plaque morphology of two strains of dengue type 1 LLC-MK_2 cells stained with neutral red. Both strains of virus were isolated from Aedes pseudo-scuttellaris cell culture harvest submitted by the Caribbean Epidemiological Center. The original serum specimens came from the Bahamas.

importance between strains of virus of the same serotype (Russell and McCown, 1972).

The principle disadvantages of this technique concern the requirements for careful cell culture husbandry and a minimum time of two weeks for virus identification. However, if one suspects that there might be sufficient infectious virus in the original serum to do a PRNT, as is frequently the case on the first day of illness, identification can be accomplished in only one week by using the original serum as a seed virus.

It has also been contended that the plaque assay may be a less sensitive method for dengue virus isolation than other procedures, such as mosquito inoculation (Rosen and Gubler, 1974). Our experience with dengue isolation from sera collected from the Caribbean in 1977 demonstrated that initial virus passage in LLC-MK_2 under liquid medium increased the number of virus isolates detected by the plaque assay procedure. Eight of 31 (26%) virus isolates from serum were obtained only after passage once or twice under liquid medium. The preliminary passage step improved our ability to detect dengue virus by increasing the concentration of virus submitted to plaque assay.

Since the specimens included in this report were submitted for a variety of reasons, they are not necessarily representative of all of the serotypes present in each country during the epidemic nor even the relative frequency of each serotype. The data do provide an indication of the distribution of dengue type 1 in 1977 and will be of value for comparison to the results of serotype identifications of the same strains by other methods.

Low passage dengue isolates from the Caribbean did not give strong homotypic reactions with the types 1 and 2 reference ascitic fluids. In a few cases, additional passage of the virus improved the neutralization reactions. The consistent low titers of dengue type 1 ascitic fluid required to neutralize the Caribbean strains present the possibility that these viruses differ significantly in antigenic composition from the Hawaii reference strain. In an analogous situation, strains of dengue type 3 from Tahiti and the Caribbean were found to be antigenically different from strains from Southeast Asia.

In our experience, the identification of dengue viruses by other serological techniques, such as complement fixation or direct immunofluorescence, has serious practical and theoretical disadvantages. The degree of cross reactivity between serotypes

makes it difficult to differentiate virus strains in a dependable and reproducible manner. The specificity of any identification procedure is determined by the number of antigenic components that share in the reaction, the type-specificity of each component, and the ability of the user to identify and control the multiple reactive components. The ideal test should detect the reaction of only one type-specific antigen and its corresponding antibody.

Dengue viruses, like other flaviviruses, produce three structural and at least five nonstructural proteins. The major envelope protein, V_3, has a molecular weight of 53000-59000 daltons (Westaway, 1973; Shapiro et al., 1972), and contains both group- and type-specific antigenic determinants (Qureshi and Trent, 1973; Della-Porta and Westaway, 1977). Neutralization tests measure only the reaction between the antigenic determinants on the surface of the virion and the corresponding antibodies. For flaviviruses the reaction involves only the V_3 protein. Serologic procedures that do not depend upon viral infection, such as complement fixation, immunofluorescence, and radioimmunoprecipitation, detect reactions involving several structural and nonstructural protein antigens and the corresponding antibodies. Since some of the antigens are broadly cross-reactive, these tests cannot be entirely type-specific. Antibodies prepared to all of the viral specified proteins found in infected suckling mouse brain and cell cultures may be highly specific in neutralization tests but tend to be cross-reactive when used in other serologic procedures. For reasons related to the number and the specificity of antigens involved in each test procedure, the plaque reduction neutralization test is more specific and reliable than other serotyping methods.

The ideal dengue virus isolation system for remote field studies may require a combination of initial virus isolation in a conveniently transportable and sensitive assay procedure, such as mosquito inoculation or mosquito cell culture followed by plaque assay and plaque reduction neutralization test in LLC-MK_2 cells for studying virus growth characteristics and obtaining highly specific virus identification. Future studies of dengue in the Caribbean might incorporate a definitive study of these combined virus isolation techniques.

F. Effect of Ribavirin on Dengue-1 Replication

Ribavirin is an effective antiviral drug with *in vitro* and *in vivo* inhibitory activity against a broad spectrum of DNA and RNA viruses (Simon et al., 1973; Huffman et al., 1973; Witkowski et al., 1972; and De Clercq et al., 1975). Several togaviruses, including sindbis, Semliki Forest, and Japanese encephalitis virus, have exhibited moderate susceptibility (minimal inhibitory concentration approximately 30 mcg/ml) to the antiviral effect of Ribavirin. These results prompted an investigation of the effect of Ribavirin against *in vitro* dengue virus infection.

1. Method

All experiments used monolayers of LLC-MK2 cells in 25 cm^2 plastic flasks infected with dengue type 1 virus (Western Pacific strain) at an MOI of 0.1 pfu/cell. Virus production was quantified by a standard plaque assay of supernatant fluid collected on days 1, 2, 5 and 7.

Concentrations of Ribavirin ranging from 0.1 to 100 mcg/ml were tested in three ways. First, the cytotoxic effect of Ribavirin was determined by exposing uninfected cell cultures to the drug for 7 days. Cell growth and morphology were observed daily.

The effect of preincubation of cells with Ribavirin before infection on virus replication was studied by exposing cells to Ribavirin for three hours, washing cells with Earles balanced salt solution and incubating them in medium 199 with 20% fetal bovine serum.

Finally, the effect of continuous exposure from 3 hours before infection throughout the course of the infection was studied.

2. Results

No noticeable Ribavirin cytotoxicity was evident from microscopic examination of the control monolayers until the 7th day of the experiment, when a small decrease in the percentage of viable cells was noted. Figure 8 shows virus growth curves from the pretreated washed cells. No significant inhibition of virus production was evidenced by pretreatment with 0.1, 0.5, 1.0, 5.0 or 10 mcg/ml Ribavirin for 3 hours before infection. A

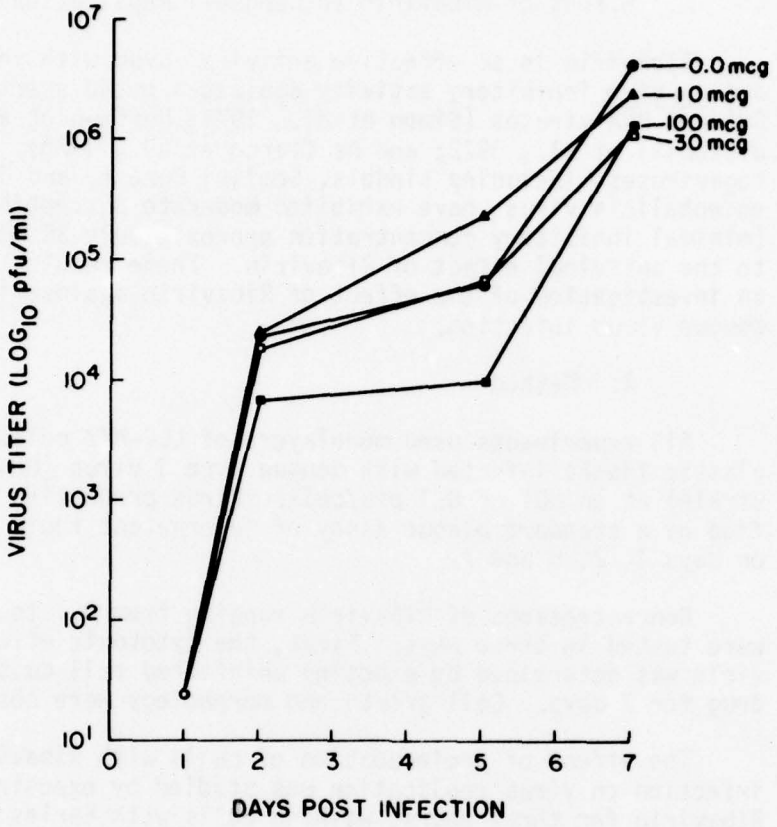


Fig. 8. Growth curves of dengue type 1 following pre-treatment for 3 hours with various doses of Ribavirin.

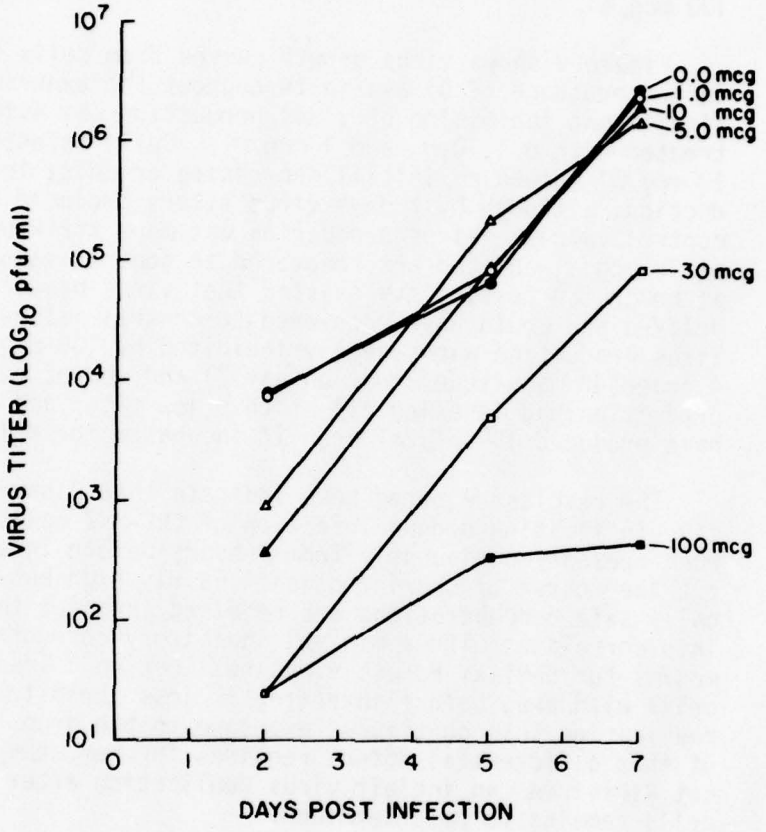


Fig. 9. Growth curves of dengue type 1 with continuous treatment with varying doses of Ribavirin starting 3 hours before infection and lasting until day 7.

slight inhibition in final titer on day 7 is suggested at 30 mcg/ml; while the entire growth curve is slightly depressed at 100 mcg/ml.

Figure 9 shows virus growth curves from cells incubated in the presence of Ribavirin throughout the experiment. No significant inhibition of virus production was evident in cells treated with 0.1, 0.5, and 1 mcg/ml. Cells infected with 5 and 10 mcg/ml showed an initial depression or delay in virus production, although by 7 days virus titers produced are equal to control values. Virus production was more strikingly depressed at 30 mcg/ml and had not recovered to control values by day 7 although the possibility existed that virus production was delayed and would have recovered to control values by day 9. Virus production was severely inhibited by 100 mcg/ml (almost 4 ordersof magnitude lower on day 7) and showed evidence that production had levelled off at this low titer and would not have produced 10^6 pfu/ml even if incubated for a longer period.

The results reported here indicate that Ribavirin is effective in limiting dengue infection of LLC-MK2 cells in vitro when present continuously from 3 hours before infection throughout the course of the infection. Fairly high but pharmacologically safe concentrations are required (greater than 10 mcg/ml). This correlates with a minimal inhibitory concentration of 30 mcg/ml for Semliki Forest virus replication. Treatment of cells with drug before infection is less inhibitory of virus replication than continuous exposure to the drug. The mechanism of this differential effect requires further study. Whether or not Ribavirin can inhibit virus replication after infection of cells remains to be determined.

II. Respiratory Viruses

A. Acute Respiratory Disease (ARD) in Military Personnel

1. Adenovirus

In recent years ARD on all basic combat training (BCT) posts has been predominantly due to adenovirus type 21. Although types 4 and 7 have been isolated on occasion, ARD due to adenovirus types 4 and 7 was well controlled by immunization with live, oral adenovirus types 4 and 7 vaccines. The type 4 vaccines (Wyeth Lots 07401 and 07701) titered 5.1 and 5.0 log 10

TCID 50 per tablet while the type 7 vaccines (Wyeth Lots 07501 and07801) titered 6.1 and 6.0 log 10 TCID 50 per tablet, respectively. Lots 07401 and 07501 for types 4 and 7, respectively, were used from the end of September 1977 until the end of February 1978 and 07701 and 07801 were introduced thereafter.

In the past, influenza has caused brief increases in ARD rates. ARD in FY 78 showed a similar pattern. Outbreaks of influenza A/USSR/77 in late winter on several posts coincided with sharp increases in ARD rates to the peak levels for the year. Within a few weeks, ARD rates on most posts had returned to the levels preceding influenza outbreak.

The impact of adenovirus infections on BCT posts was evaluated by reviewing weekly ARD rates provided by the Preventive Medicine Consultant, DASG, and virus isolation records from the four U.S. Army Medical Laboratories contributing to the adenovirus surveillance program. Dates of vaccine administration were provided by representatives of the USAMEDDAC on each BCT post.

The respiratory disease patterns on eight Army BCT posts under surveillance can be summarized as follows:

a. Fort Bliss. Trainees were immunized with type 4 and 7 vaccines from 1 Oct 77 to 31 Apr 78. Virus isolates included 2 type 7 strains and 1 type 21 strain but no type 4 strains. For eleven months ARD rates ranged from 0.0 to 4.0/100/week, but in February, rates jumped to a peak of 25/100/week.

b. Fort Dix. Adenovirus vaccines were administered from 1 Sep 77 to 30 Apr 78. Virus isolates from ARD patients included adenovirus type 4 (3 isolates), type 7 (4 isolates) and type 21 (6 isolates). ARD rates ranged from 0.3 to 2.0/100/week except for February, when they peaked at 10.9.

c. Fort Gordon. Male BCT's received adenovirus vaccination at Fort Jackson between 1 Oct 77 and 1 May 78. Females were not immunized. Male and female combined ARD rates ranged from 0.0 to 2.3 except for February when they peaked at 4.3/100/week. Virus isolates included 8 strains of adenovirus type 4, of which 7 strains were recovered in the month of May. Only one strain of type 7 virus was isolated. Sixty strains of type 21 were reovered; 42 isolates were recovered between

18 March and 30 April at a time when the ARD rate rose from 1.4 to 2.3/100/week, then fell to 0.4.

d. Fort Jackson. Adenovirus vaccines were given to male BCT's from 1 Oct 77 to 1 May 78. Combined ARD hospitalization rates ranged from 0.2 to 2.2/100/week with a peak in February of 1.7/100/week. Virus isolations from ARD hospitalizations included adenovirus type 4 (4 strains), type 7 (12 strains) and type 21 (32 strains). Twenty-six strains of type 21 were isolated between 1 April and 14 May. All but one type 4 and 7 virus strain were isolated after the close of the vaccination program.

e. Fort Knox. Adenovirus vaccines were given from 27 Oct 77 to 31 Apr 78. ARD rates ranged from 0.2 to 1.9/100/week except for four weeks starting the last week of January. The peak rate was 6.5 during the second week of February. Adenovirus type 21 was isolated each month, but one half of the 33 isolates for the year were obtained between October and January.

f. Fort Sill. Adenovirus vaccines were given from 1 Oct 77 to 31 May 78. ARD rates ranged from less than 0.1 to 0.9/100/week except for two weeks in February when the peak rate was 6.5. Only two adenovirus isolates were obtained from this post; both were type 21 viruses.

g. Fort Wood. As in previous years, adenovirus type 4 and 7 vaccines were administered all year long. ARD rates ar Fort Wood showed an early peak of 2.1/100/week in December, fell to 1.0 then rose again to 7.9 during the last week of January. Afterwards, rates remained greater or equal to 2.0 until 15 April. Adenovirus type 4 (5 strains) and type 7 (5 strains) were isolated only after 29 April. All but 6 of the 44 type 21 isolates were obtained after 25 March.

2. Influenza

A/USSR/77 spread throughout the U.S. after 1 January 1978 causing sudden outbreaks in people under age 25 years in colleges, military academies and military posts. Peak illness in most civilian populations occurred in February.

a. Fort Eustis, VA.

From 17 to 19 January 1978, the incidence of ARD reportedly

doubled at one clinic which serviced trainees at the transportation school. Examination of 18 sequential clinic visitors on 19 January revealed typical findings for influenza (Table 27). Throat wash material collected from these 18 men and 12 women yielded 8 isolates of A(H_3N_2) despite a history of recent influenza vaccination from some patients.

Table 27. Clinical Data for 18 ARD Patients at Ft. Eustis, VA.

Clinical Findings	Frequency
Symptoms	
Fever	94%
Chilliness	90%
Cough	83%
Sore Throat	72%
Headache	44%
Myalgia	39%
Chest Pain	39%
Nausea	33%
Diarrhea	28%
Abdominal Pain	28%
Signs	
Pharyngitis	100%
Cervical Adentitis	11%
Wheezing	4%
Rhonchi	4%
History	
Mean age	21 years
Recent influenza vaccine	77%

b. USA Military Academy, West Point, N.Y.

Between 23 January and 7 February 1978, 2602 of 4400 cadets (59%) reported on sick call with respiratory illness; 14 required admission to the hospital. During the same time interval in 1977, there were 600 sick call visits and 42 hospital admissions for respiratory disease. Although attack rates were higher in 1978, the influenza in 1978 seemed to cause a relatively mild disease in comparison to earlier outbreaks. In 1978, the typical syndrome included fever 100-101°F, mild cough, mild headache and severe muscle aches. Pneumonia was rare. Throat wash specimens collected from 30 people on 31 January yielded four isolates of influenza A (H_1N_1)

c. Other Military Posts

Isolates of influenza A (H_3N_2) were recovered from specimens from Ft. Wood received on 7 Dec 77. H_1N_1 isolates were found in specimens received from Aberdeen Proving Ground, MD (3 Feb 78) and Ft. Dix (8 Feb 78).

B. The Use of Transformed Human Embryonic Kidney Cells for Adenovirus (ADV) Neutralizing Antibody Assays.

Since 1976 when primary human embryonic kidney (HEK) cell cultures became scarce, attempts have been made to develop an alternative cell line of equal or greater sensitivity and specificity for growing adenoviruses.

Recently Graham et al (1977) succeeded in transforming human embryonic kidney cells into an apparently continuous cell line which preserved sensitivity for adenoviruses (here in after referred to as HEK-T cells). This cell line was evaluated as a substitute substrate for assay of adenovirus neutralizing antibody in a micro-culture system.

1. Materials and Methods: Prototype strains of human adenoviruses likely to be encountered in a military population (Adenovirus types 2, 3, 4, 5, 7, 11, 14, and 21) were obtained from the American Type Culture Collection. Working stocks of virus were prepared by passing the reference virus once in monolayers of HEP-2 cells in 32 oz bottles, and harvesting at the appearance of 4 + CPE by freezing and thawing the infected cells in their growth media three times. Working stocks so prepared contained 10^7 to 10^9 $TCID_{50}$/ml (on second passage

HEK cells).

Cells: No primary human embryonic kidney cells were available during the study period. Second passage, once frozen human embryonic kidney cells (HEK-P2) were obtained commercially in screw capped tubes from Microbiologic Associates, Bethesda, Maryland. "Flow 4000" cells, the 15th to 16th passage of primary HEK cells, (HEK-P15) fibroblastic in appearance, were obtained commercially in screw capped tubes from Flow Laboratories. HEK-T cells, 45th passage, were kindly provided by Dr. Maurice Green of St. Louis University, and subsequently 25th passage HEK-T cells were kindly provided by Dr. F. Graham of McMaster University. HEP-2 cells were obtained from Microbiologic Associates. HEK-T cells were grown in monolayers in 32 oz glass prescription bottles or 150 cm^2 plastic flasks (Falcon). Weekly the cells were suspended in 3-4 ml of 0.02% EDTA in calcium and magnesium free phosphate buffered saline, and split 2:1 or 3:1. Growth medium was Eagle's minimum essential medium, Earle's base (EMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 microgram/ml of streptomycin, and 100 unit/ml of penicillin. When required for tube titrations, HEK-T cells were pipette dispensed into glass screw capped tubes; approximately 20,000 to 40,000 cells/ml/tube. Cell sheets in the tube approached confluence in 4-5 days.

Reference Antisera: Antisera to ATCC reference virus strains of adenovirus 4, 7 and 21 were prepared by immunization of New Zealand white rabbits with cesium chloride density-gradient purified, concentrated virions . Each rabbit received four subcutaneous injections of 1.0 ml containing 100 micrograms of partially purified virus and 50% complete Freund's adjuvant. After a booster injection at 21 days, rabbits were bled for sera at day 28 to 35.

Micro-cultures: For both titrations of virus ($TCID_{50}$) and serum neutralization, confluent monolayers of cells were suspended in versene-PBS (HEK-T) or 0.25% trypsin (HEP-2 cells), pelleted, and resuspended in growth media. Cell clumps in the suspension were dispersed by vigorous pipetting. Between 10 to 20 x 10^3 cells in 100 microtiters of growth media were dispensed manually into each well of sterile 96 (12 x 8) well polystyrene plates with hard plastic covers (Linbro). Cells were dispensed with autoclave sterilized microdroppers (Linbro). The plates were incubated at 35°C in 5% CO_2. Control and back-titration plates were examined daily for CPE through an inverted microscope. When indicated by experimental design, the plates were

fixed and stained by immersion in a solution of 5.6% formaldahyde and 0.07% crystal violet in water for 2 hours, rinsed by repeated gentle immersion in tap water, and allowed to dry in the air.

2. Results

Comparison of HEK cell types: Growth curves of adenovirus 2, 3, 4, 5, 6, 7, 11, 14, 21 were performed on HEK-P2, HEK-P15, HEK-T cells in confluent monolayers in glass tubes, in order to assess the permissiveness of each cell type under standardized conditions. Serial 10-fold dilutions of working stocks of each reference virus were made in EMEM 5%, the growth media was poured off the cell monolayers, and 0.2 ml of each virus dilution (10^{-3} through 10^{-7} in initial trials and 10^{-3} through 10^{-9} in later trials) was added to each of three tubes per cell type. Each cell type was infected 5 to 7 days after planting, when the monolayers were nearly confluent. Tube caps were resecured and the tubes were incubated in racks at 36°C. The medium in each tube was replaced every seven days.

Each tube was examined microscopically for CPE at frequent intervals, and the apparent $TCID_{50}$/ml was calculated for each cell type and plotted. In initial trials for some virus-cell combinations the maximum dilution of virus tested (10^{-7}) showed CPE before an asymptote was reached. Repeat growth curves, which included higher virus dilutions, showed clear asymptotes.

As depicted in the growth curves in Figure 10 and Table 28, HEK-T cells were as permissive to the development of CPE for most adenovirus serotypes as were HEK-P2 cells. Notable exceptions were adenovirus type 3, for which HEK-P2 was superior and adenovirus type 4, for which HEK-T was superior. Similar comparisons between HEP-2 cells and HEK-T cells were difficult to read consistently because of excessive rounding of cells in uninfected tubes of HEP-2's. However, using four + CPE as endpoint, HEK-T cells were regularly 10 to 100 times more sensitive for all adenovirus serotypes tested.

Micro-neutralization tests with rabbit hyperimmune sera: The specificity of virus neutralization on HEK-T cells in microculture was checked by measuring the homologous and heterologous titer of rabbit hyperimmune sera with adenovirus types 4, 7, and 21. 50 ul of growth media was added to all wells of the plates, and 50 ul of a 1:5 dilution of heat inactivated (56° x 30 min) rabbit sera was added to wells in the first row. Serial two-fold

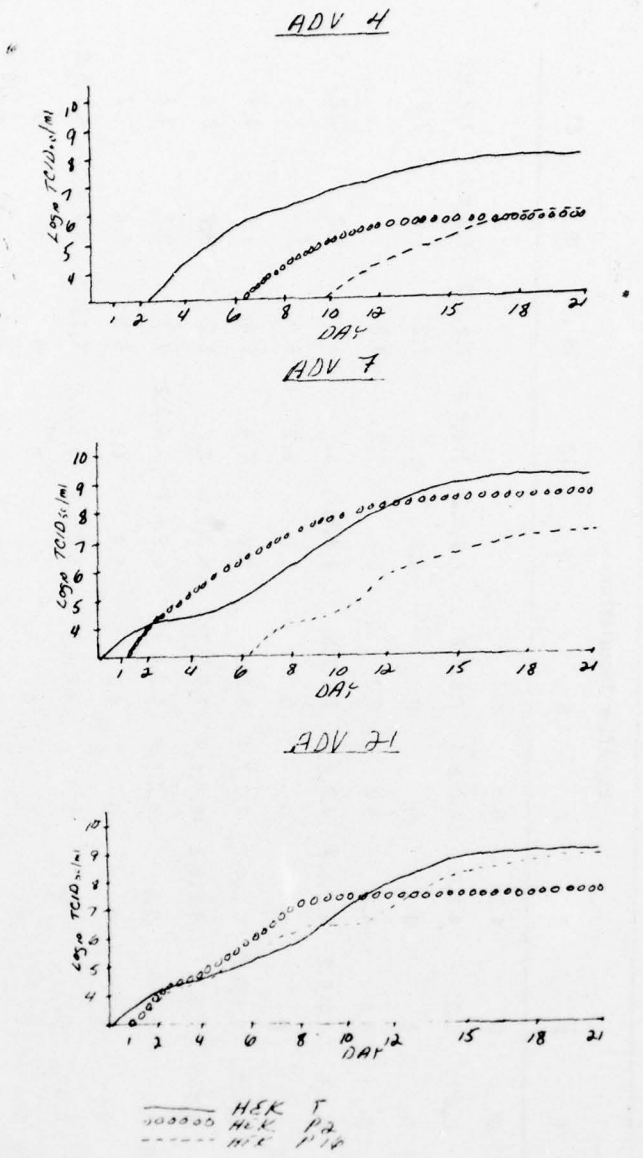


Fig. 10. Growth curves for adenovirus types 4, 7 and 21 in three cell lines; ●——●, HEK-T; ○○○○○, HEK P2; ----, HEK P16.

Table 28. Growth Curves of Adenovirus Types 2, 3, 5, 11, and 14 in Three Types of HEK Cells

ADV type	Cell type	Day After Inoculation 1	2	4	6	8	10	12	15	18	21
2	HEK-T	0*	0	4.2	6.9	8.2					
	HEK-P2	0,0	0,0	4.2,0	6.2,6.4	7.2,7.2	7.2,8.2	7.2,8.2	7.2,8.2	7.2,8.2	7.2,8.2
	HEK-P16	0	0	0	0	0	5.2	7.2	7.2	7.2	7.2
3	HEK-T	0	4.2	4.2	5.2	6.2	6.2	7.2	8.2		
	HEK-P2	0,4.5	4.2,5.2	5.2,5.9	7.2,6.4	7.2,7.5	8.2,8.2	8.2	8.2	8.2	8.2
	HEK-P16	0	0	0	0	0	5.2	6.9	6.9	7.9	7.9
5	HEK-T	0,4.2	4.5,5.2	6.2,6.2	7.7,7.7	8.2,8.2	8.9	9.4	9.4	9.4	9.4
	HEK-P2	0,0	0,0	4.2,5.2	6.4,6.9	7.9,8.2	8.4,8.2	8.2	8.2	8.2	8.2
	HEK-P16	0,0	0,0	0,0	4.2,4.2	4.2,4.9	6.2,6.7	7.4,8.2	7.7	8.2	8.2
11	HEK-T	0	0	0	0	4.2	5.2	6.2	6.9	7.2	7.2
	HEK-P2	0,0	0,0	0,0	0,0	4.2,4.9	5.2,5.9	5.5,5.9	5.7,5.9	5.7,5.9	5.7,5.9
	HEK-P16	0	0	0	0	0	0	0	0	0	0
14	HEK-T	0	0	4.2	4.5	6.2	6.2	6.4	7.2	7.2	7.2
	HEK-P2	0,0	0,4.2	5.2,5.4	5.2,5.9	6.2,6.2	6.4,6.9	6.4,6.9	6.4,6.9	6.9,6.9	6.9,6.9
	HEK-P16	0	0	0	0	0	0	0	4.2	4.7	5.2

*Log 10 apparent $TCID_{50}$. Titers $<10^{3.4}$/ml are indicated by 0.

dilutions were made with hand-held micro-diluting loops, which were twirled gently to avoid excessive abrasion of bottom surfaces of the wells. 50 ul of a 1:1000 dilution of the working stock of reference viruses were added to each well. The plate was agitated gently by tapping, and incubated for two hours at 36^0 in 5% CO_2 before 100 ul of cells were added per well. Each plate included a cell control row (no added virus) and a virus control row (no added serum). Each serum dilution was tested in three wells. Neutralizing antibody titers were expressed as the maximum serum dilution in which all three wells had no detectable CPE. Table 29 shows that even with the very low challenge doses used, only homologous virus was neutralized.

Table 29. Specificity of Adenovirus Neutralizing Antibody Assay in HEK-T Microculture.

Virus Serotype*	Rabbit Hyperimmune Serum ADV-4	ADV-7	ADV-21
4	≥320**	<10	<10
7	<10	≥320	<10
21	<10	<10	≥320

*Challenge dose was 3 $TCID_{50}$/well.
*Reciprocal serum titers read at 48 hours for ADV-7 and 21 and 96 hours for ADV-4.

Micro-neutralization test with serum from recruit vaccinees: Forty-seven paired serum specimens from recruits at Lackland AFB, who had received triple (adenovirus 4, 7, and 21) live enteric immunization (N=33) or double (adenovirus 4 and 7) live virus enteric immunization (N=14) were tested. These specific sera were selected because they had been tested for adenovirus neutralizing antibody by the tube methods with primary HEK cells before this type of test became unavailable. Paired sera were run simultaneously by the micro culture method using HEK-T cells. Dilutions of serum, 1:2 through 1:64, were prepared in 50 ul volumes in the wells, and 50 ul of each virus suspension were added (1:300, 1:1000, 1:50 for adenovirus 4,7,

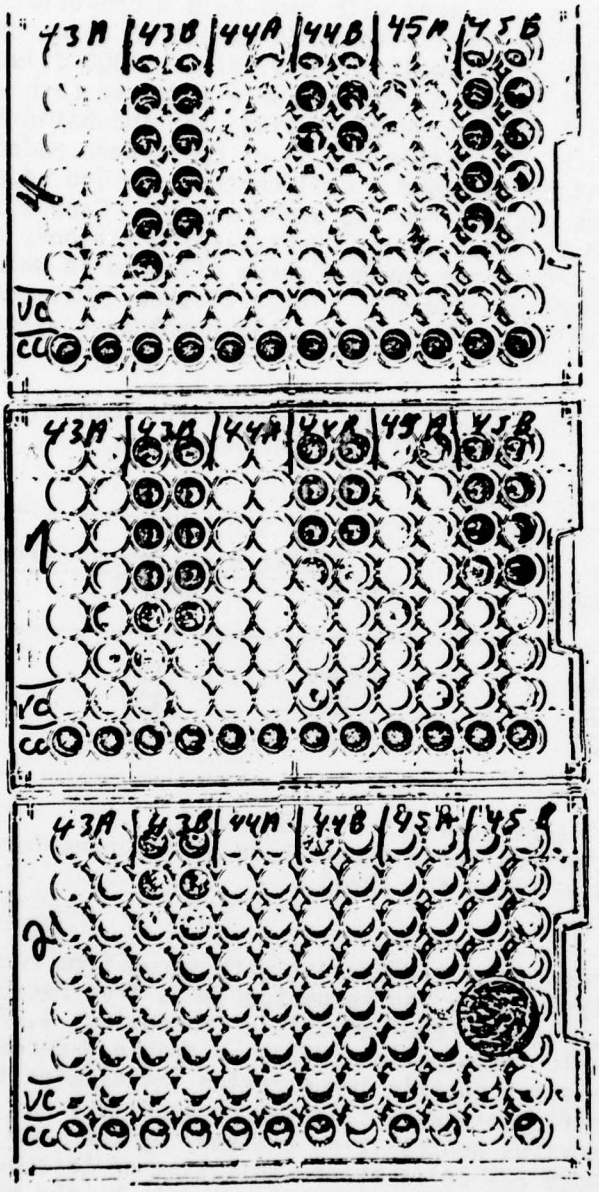


Fig. 11. Adenovirus microtiter neutralizing antibody assay for three serum pairs using adenovirus types 4, 7 and 21. vc is virus control; cc is cell control.

and 21 stock, respectively). Results were read microscopically and after fixing and staining on Day 5. Titers were read from the stained plate as the highest dilution with greater than 50% preservation of the cell sheet. Sample plates showing the results for 3 serum pairs are presented in Figure 11. Comparison of the titers obtained by HEK-T micro-neutralization test to the primary HEK-tube method are presented in Figure 12. From this data it can be seen that there was an excellent correlation between the two cell culture methods for adenovirus type 4 and type 7, but that the titers by micro-culture neutralization for adenovirus 21 were much lower than those previously obtained by tube culture. Interpretation of the data at this point was difficult because the serum specimens tested had been obtained from subjects in a vaccine trial to test for possible interference of adenovirus type 4 and 7 immunization on the serum antibody response to adenovirus 21 immunization. Either of two possibilities was equally plausible: 1) The tube method ADV titers were dtermined in primary HEK cells with reduced sensitivity to the virus, or 2) the micro-culture ADV 21 neutralization titers were spuriously low. We therefore, cursorially examined the effect of virus dilution on the neutralizing antibody titers determined in the HEK-T micro-cultures and evaluated the test with more rigorously defined control groups.

Effect of virus dilution on SN titer: Convalescent serum from a recruit immunized with ADV 4,7, and 21 and previously shown to have a high titer of type 21 neutralizing antibody was tested. Methods were those outlined above, except that several dilutions of working virus stock were tested (1:10, 1:30, 1:100, 1:300, and 1:1000). Wells were examined microscopically for CPE. The neutralizing antibody titer was defined as that dilution showing greater than 50% preservation of the cell sheet compared to that present in the virus-free control wells. Results are depicted in Table 30. Even at the same "$TCID_{50}$" challenge dose, lower dilutions of virus gave lower neutralizing antibody titers.

Micro neutralization of serum specimens from recruits with adenovirus 21 acute respiratory disease: During studies of acute respiratory disease at Ft. Dix, 1976, nine recruits were studied who fulfilled the criteria of (1) a pre-illness survey blood specimen (2) a ten-day post illness blood specimen (3) symptomatic acute respiratory disease and (4) isolation of adenovirus 21 from throat washings. Micro neutralization titers were done on the pre-illness and post-illness serum specimens. Serum

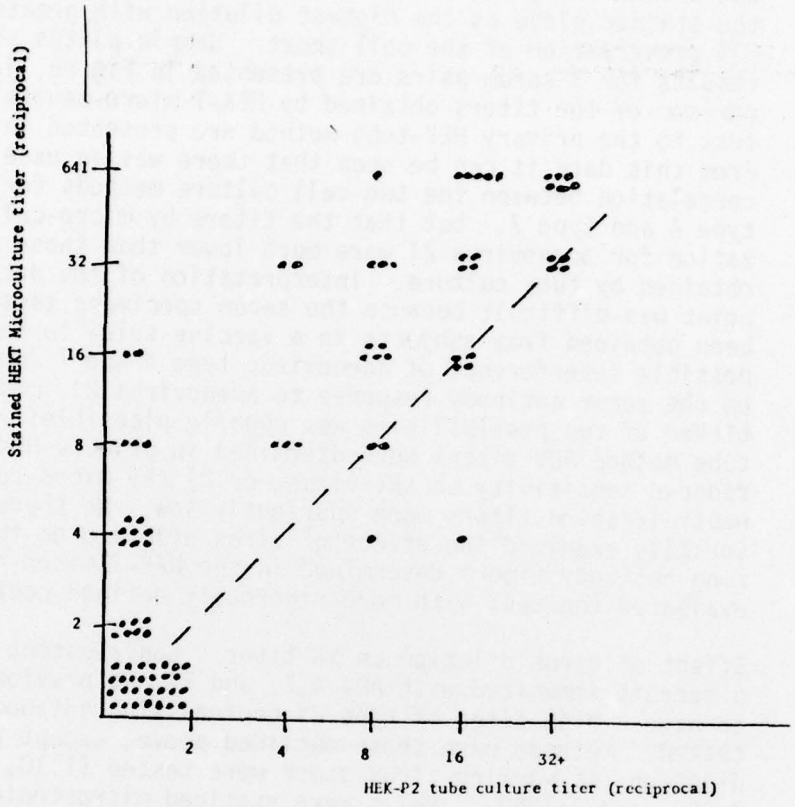


Fig. 12a. Adenovirus type 4 neutralizing antibody titers determined by HEK-T microculture method compared to titers determined by the HEK-P2 tube method.

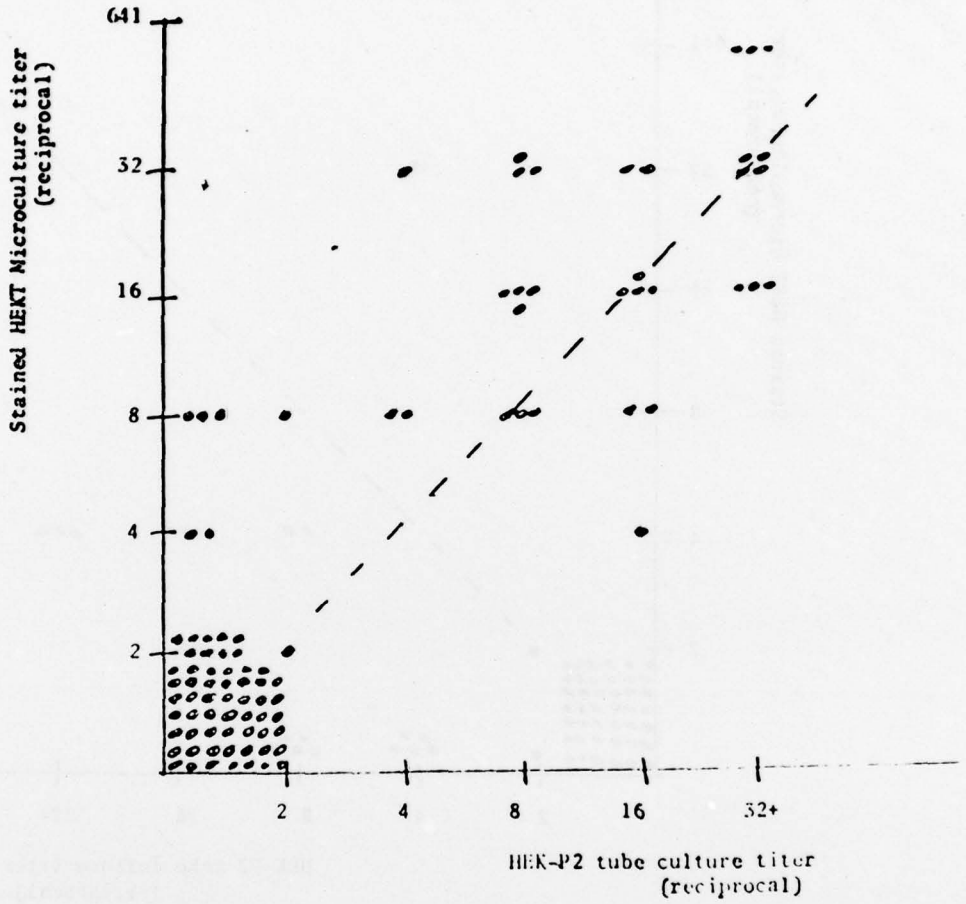


Fig. 12b. Adenovirus type 7 neutralizing antibody titers determined by the HEK-T microculture method compared to titers determined by the HEK-P2 tube method.

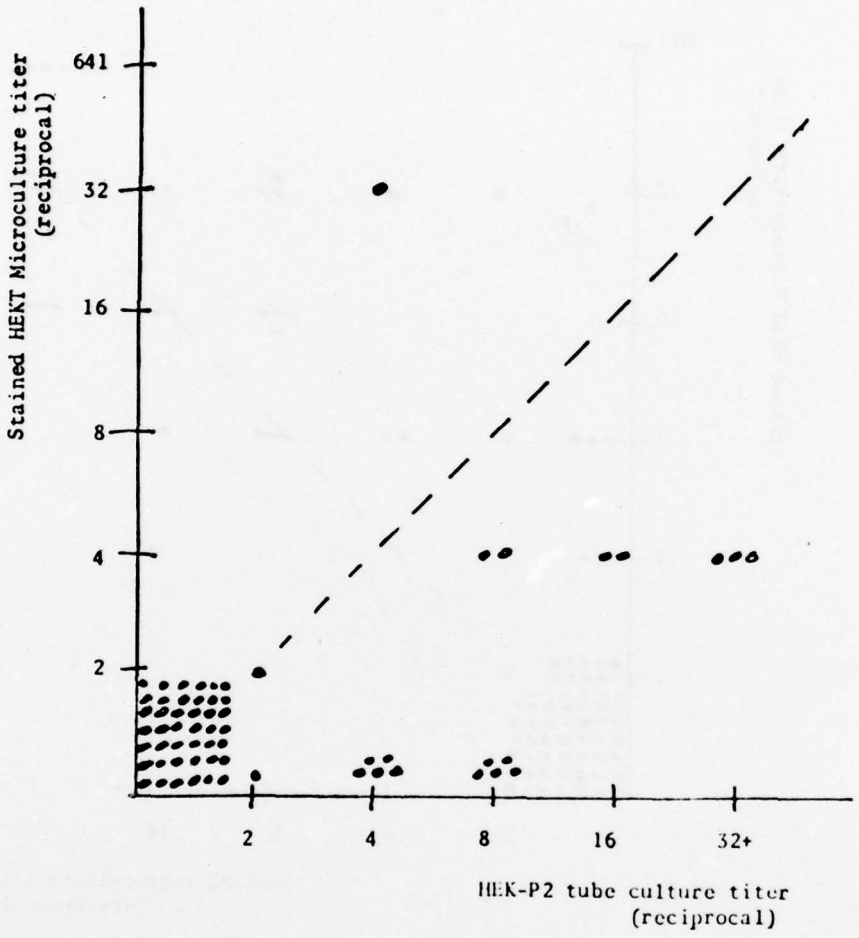


Fig. 12c. Adenovirus type 21 Neutralizing antibody titers determined by the HEK-T microculture method compared to titers determined by the HEK-P2 tube methods.

Table 30. Relationship of Virus Dilution and Time of Reading to Neutralizing Antibody Titer.

Hours Post Inoculation	Virus Dilution				
	1:10	1:30	1:100	1:300	1:1000
12	1:8(10)*	1:16(3)	N**	N	N
36	1:2(100)	1:4(33)	1:16(10)	1:32(3)	N
60	1:2(300)	1:4(100)	1:16(30)	1:32(10)	1:64(3)
84	<1:2(1000)	1:2(300)	1:8(100)	1:16(30)	1:64(10)

*Serum neutralizing antibody titer (virus $TCID_{50}$ per well).
**No CPE to be neutralized.

pairs from six of the nine patients showed a four fold or greater rise in titer; the convalescent geometric mean titer of the seroconvertors was 1:16.

Micro neutralization of serum specimens from immunized recruits: Serum pairs from four groups of 10 men each who were immunized in the Lackland AFB trial were tested for neutralizing antibody to ADV 21 bythe HEK-T micro-neutralization method. Immunizations given to each group were as follows: Group A received ADV 4, 7, and 21; Group B received no adenoviruses. Results are depicted in Figure 13. Of volunteers with pre-immunization titers of $\leq$ 1:4, 3 of 8 in group A, 5 of 8 in group B, 0 of 9 in group C, and 0 of 8 in group D showed a four-fold or greater rise in titer. The geometric mean titers of seroconvertors in group A was 1:13; and group B, 1:14. Forty-four serum specimens of the 40 serum pairs had previously been titered by the primary HEK tube method. Values are plotted in Figure 14.

3. Discussion

Testing a new method for measuring adenovirus neutralizing antibodies in human serum in the absence of primary human embryonic kidney cells proved to be awkward; since we were unable to measure titers simultaneously by both methods. Instead we were forced (1) to compare our data to data previously obtained

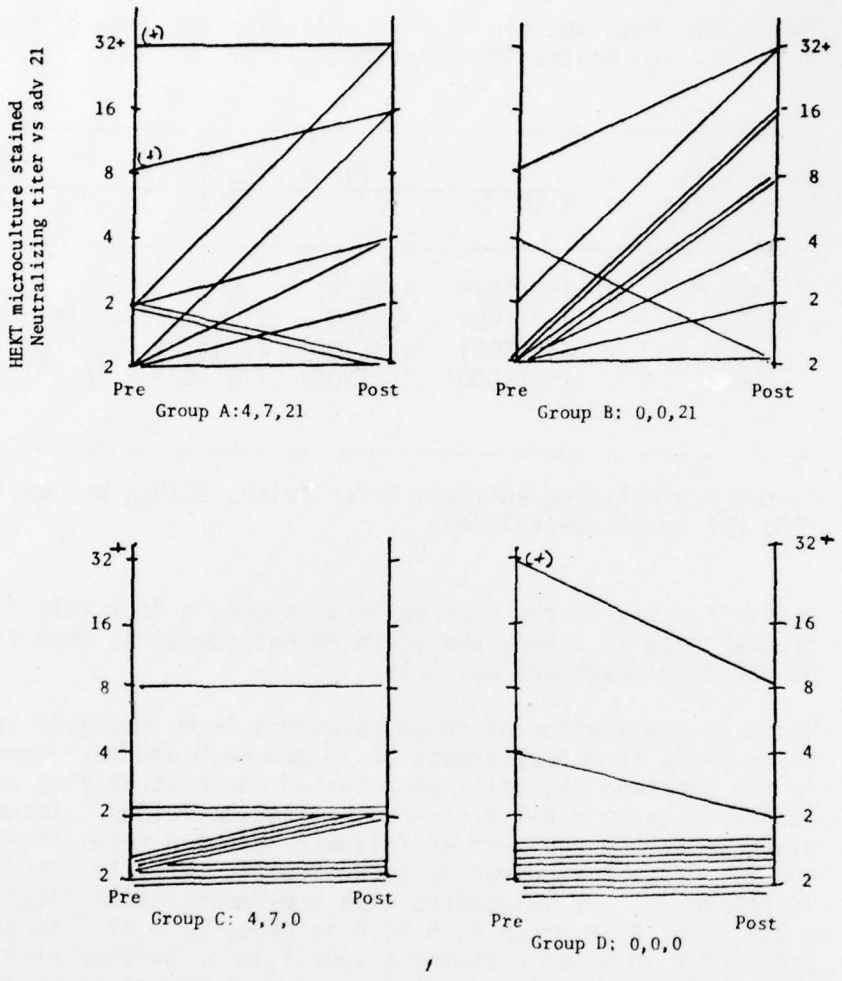


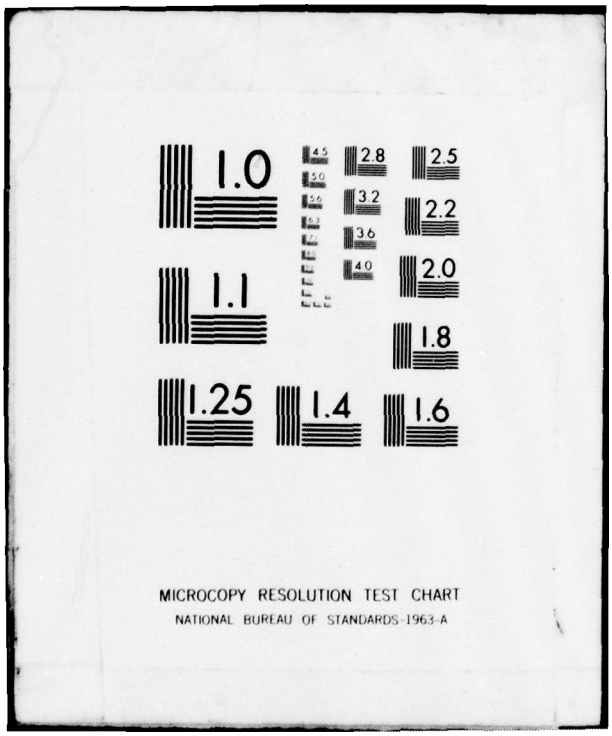
Fig. 13. Changes in serum adenovirus neutralizing antibody titers in four vaccine groups at Lackland AFB. Vaccines administered are indicated beside the group letter. Pre and post serum samples were collected on Days 0 and 21, respectively. (+) denotes sera positive by tube neutralization test also.

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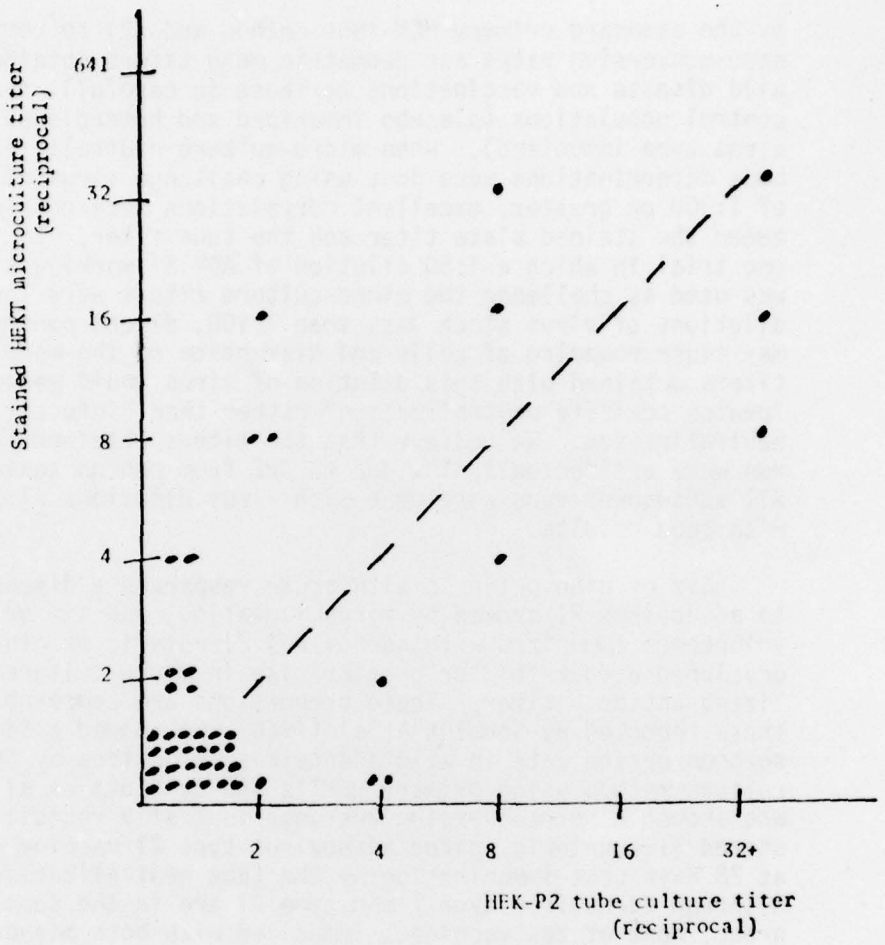


Fig. 14. Adenovirus type 21 neutralizing antibody titers by the HEK-T microculture method compared to titers determined by the HEK-P2 tube method. Serum specimens were obtained from Lackland AFB vaccine study, 1976.

by the standard primary HEK-tube method and (2) to compare the sero-conversion rates and geometric mean titers obtained in wild disease and vaccinations to those in carefully selected control populations (placebo immunized and heterologous virus type immunized). When micro-culture neutralizing antibody determinations were done using challenge virus dilution of 1:300 or greater, excellent correlations were obtained between the stained plate titer and the tube titer. In the one trial in which a 1:50 dilution of ADV 21 working stock was used as challenge the micro-culture titers were low. At dilutions of virus stock less than 1:100, direct penton toxicity may cause rounding of cells and disruption of the monolayer; titers obtained with this dilution of virus would measure "penton toxicity neutralization" rather than "infectivity" neutralization. We believe that the titers obtained in this run were artifactually low due to CPE from penton toxicity. All subsequent runs were done with virus dilutions $\geq$1:300, with good results.

Six of nine patients with acute respiratory disease due to adenovirus 21 proven by virus isolation, and six or seven volunteers immunized with adenovirus 21 enteric vaccine developed a four-fold or greater rise in micro-culture nautralizing antibody titer. These proportions are comparable to those reported by Schmidt et al (1966) who showed a 54-94% seroconversion rate in wild adenovirus infections by the micro-culture method using primary HEKT's and by Scott et al (1972) who showed a seroconversion response in 7 of 9 recruits administered live enteric coated adenovirus type 21 vaccine as tested at 28 days post-immunization by the tube neutralization method. Although adenovirus type 7 and type 21 are in the same typing group, none of ten vaccinees immunized with both adenovirus 7 and adenovirus 4 showed a neutralizing antibody response to adenovirus 21, and adenovirus 7 hyperimmune rabbits showed no neutralization of adenovirus 21; both tests confirmed the specificity of micro-culture method.

We believe that the micro-culture of transformed human embryonic kidney cells are the optimal method for determination of adenovirus neutralizing antibodies in serum surveys and vaccine studies. Transformed human embryonic kidney cells are highly permissive for growth of most common adenovirus types, the cells grow rapidly with standard cell culture techniques, and they form a smooth, solid adherent monolayer through at least 60 passages. The micro-culture method is simpler, more efficient, and less expensive than similar

titrations performed in tubes. We are now using this method as a means of assessing immunity to adenovirus in vaccine and epidemiological studies. Investigation is needed to evaluate the usefulness of the HEK-T cell line for the isolation of adenoviruses from clinical specimens.

C. Adenovirus type 21 (ADV-21) vaccine

1. Background

In the ADV-21 Vaccine Trial at Ft. Dix, N.J., (in November 1976) (1977 Annual Report) some recipients of the vaccine developed ARD due to ADV-21. There was concern about the immunogenicity of the vaccine given to the troops. Although there was adequate virus in ADV-21 vaccine tablets tested prior to the vaccine trial, evaluation of the antibody response post-immunization was incomplete due to a lack of satisfactory primary human embryonic kidney cells, which were required for adenovirus neutralization tests. The development of a transformed human embryonic kidney cell line (HEK-T) and its establishment as an alternative cell line for adenovirus neutralization tests, permitted an evaluation of the immune response to vaccination.

2. Method

At Ft. Dix, two groups of 300 volunteers each were selected, based upon the last digit of an assigned study number. Vaccine administration was double blind, with one group receiving ADV 4/7/21 vaccine and the other receiving ADV 4/7 and a placebo (P). ADV-21 vaccine lot 98A2875 titered $10^{5.7}$ $TCID_{50}$. Paired sera were collected from all volunteers at the time of vaccination (Day 0) and three weeks later (Day 21).

To determine the immune response to vaccination, subjects in each group were randomly selected for study. Day 0 and Day 21 sera were tested in duplicate using a micro-neutralization test with HEK-T cells. Of 185 volunteers tested, 83% were seronegative at 1:4 on Day 0 (Table 31). No difference was found in the frequency of seroconversion to ADV-21 between the immunized and the nonimmunized groups. This indicated that the ADV-21 vaccine, as used in this study, may not have been immunogenic in these volunteers.

The same lot of ADV-21 vaccine was given to Air Force

volunteers at Lackland AFB. Immunization with ADV-21 alone and simultaneously with type 4 and type 7 vaccines resulted in seroconversion rates of 70% and 59%, respectively, at a 1:4 dilution by tube neutralization test. The apparent lack of immunogenicity of the type 21 vaccine at Fort Dix is not readily explained, but three possibilities remain. First, the HEK-T microtiter neutralizing antibody assay may be less sensitive than the previously available primary HEK tube neutralizing antibody test. This is supported by preliminary comparative data presented in Figure 12. Even so, it is surprising that there were not more seroconversions in the vaccinee group. Second, the vaccine may have lost potency before administration. Third, triple vaccination with type 4, 7 and 21 vaccines may have caused a greater interference with the immune response to ADV-21 than was apparent in the earlier study at Lackland AFB.

D. Investigation of Murine Adenovirus Teratogenicity in Guinea Pig Embryos.

1. Background

In order to determine if murine adenovirus is teratogenic, it is necessary to find an experimental animal model in which the embryo is susceptible to virus replication but the acute virus infection does not have a fatal outcome for the embryo or fetus. This requirement eliminates the mouse as a model system because in embryonic and newborn mice, adenovirus infection is fatal. It was decided to try to infect guinea pig embryos with murine adenovirus for use as an animal model.

2. Method

Four or five female guinea pigs were housed together in wire bottom cages. A male was placed in each cage in the afternoon. The following morning the females were checked for the presence of a copulation plug (a firm, white vaginal coagulum), which indicated that copulation had occurred. Vaginal plugs in guinea pigs often fall out of the vagina, but can be easily seen in the bedding tray beneath the wire bottom of the cage. Inspection can easily determine which females have copulated.

The guinea pig estrous cycle lasts for 15 to 20 days with estrous lasting for 12 hours. Only during this 12-hour period

Table 31. Adenovirus Type 21 Neutralizing Antibody Response of Ft. Dix Volunteers, 1976.

Vaccine Group	Volunteers Studied	Pre-vaccine titer <1:4		Seroconversions ≥1:8	
		No.	(%)	No.	(%)
ADV-4/7/21	95	83	(87)	10	(12)
ADV-4/7/P	90	71	(79)	7	(9.8)
TOTAL	185	154	(83)	17	(11)

of estrous will the female copulate and, if they do, they invariably become pregnant. After copulation is detected, the pregnant females were caged separately until the desired stage of pregnancy was reached.

Four pregnant guinea pigs, 20 days post fertilization, were anesthetized, and submitted to laporotomy. Murine adenovirus was injected into the amniotic sac of the embryo with a 27 gauge needle extending through the uterine wall. The maternal abdomen was closed and the animal recovered from anesthesia. The inoculum for each embryo was 0.1 ml containing 100 mouse LD50 as determined in newborn mouse titrations. Previous titrations had determined that death and maximum virus titers were obtained on Day 10 after injection.

On Days 3, 6, 9 and 12 after inoculation of the embryos, a maternal animal was killed and the embryos removed for virus assay in newborn mice.

3. Results and Discussion

No virus was isolated from any of the embryos. It is concluded from these experiments that guinea pig embryos are not an appropriate animal model system to test the teratogenic

effects of murine adenovirus. Any attempts to test this virus or any other for teratogenicity should be tested in an animal model system that permits non-lethal replication of the virus in the embryo under investigation.

III. Interferon

Interferons are a group of glycoproteins made by a wide variety of cells in response either to viruses and synthetic polynucleotides or to immune stimulation both by intracellular inhibition of virus and by immune mechanisms. Interferon is felt to have an important role in host defense against viral infections. Although interferons are active against many viruses, they are relatively species specific in protection; generally cells are most sensitive to interferon made by cells from the same species. This necessitates development of different assays for interferons from different species. To support studies of the mechanism of Semliki Forest Virus (SFV) virulence in mice and to investigate the mechanism of interferon induction, a mouse interferon assay was developed.

A. Methods

1. Mouse dye uptake interferon (IF) assay

Methods for assaying interferons have been adequately discussed in several recent reviews (Finter, 1973; Buckler, 1977). The dye uptake method is considered highly precise and convenient and moderately simple in technique. As such, it compares favorably with other methods. Thus, a semi-micro dye uptake technique has been adapted for use as the primary interferon assay in this laboratory (Borden, 1977).

a. General description of assay

Cells are inoculated into 2 ml wells on a 96-well tray (Linbro, Hamden, Conn.) At a confluency growth media is removed and serial dilutions of interferon are added. Interferon dilutions are prepared in Hanks Balanced Salt Solution (HBSS) in medium 199 plus 2% fetal calf serum (FCS) with an Oxford automatic pipetter. 0.5 ml of each dilution is added to each of 2 wells. After 20-24 hours at 37°C in a CO_2 incubator, interferon samples are aspirated, the cells washed once with HBSS (1.0 ml) and challenge virus (0.5 ml) in HBSS-199 with 2% FCS added. At 90-95% CPE in viral control wells (48 hours), virus is removed and the remaining viable

cells stained with 3.2% neutral red in Earles Balanced Salt Solution (EBSS) for 75 minutes at 37°C. The supernatant is then removed, the cells washed once with EBSS, and the intracellular dye eluted with 1.5 ml solution of absolute ethanol and 0.1 M NaH_2PO_4 (1:1 solution). The eluted dye is measured colorimetrically at 540 mu on a Gilford Stasar II spectrophometer. The interferon titer is the inverse of that dilution at which 50% of the cells are protected: (optical density (OD) of cell control + OD viral control). In each assay, an in-house standard is titered, which has been compared to a NIH reference standard. Thus interferon titers can be adjusted to the in-house standard or to the international standard and compared between tests or laboratories.

All assays are done in a laminar airflow hood and in covered 96-well trays. Contamination is very rare. Cells and reagents are prepared and dispensed with automatic pipetters (Biorad and Cornwall) resulting in maximum precision and convenience.

b. Cells

L929 mouse fibroblasts were selected for the mouse interferon assay since these cells are of a continuous line with rapid growth characteristics. L929 cells were suspended in HBSS-199 with 2% FCS and 0.8 ml inoculated into each well of the interferon tray. Too heavy an inoculum prevented completion of the assay because of cell overgrowth and fragility of the monolayer. Too sparse a monolayer on the other hand, usually decreases the sensitivity of the test. In the range of usual seeding densities, however, the interferon titer does not significantly vary (Table 32). Over long periods of time the L929 cells have variable growth rates and therefore adjustments in seeding concentrations are needed. Currently using $2x10^5$ or $1x10^5$ cells/ml results in a confluent monolayer ready for the assay in 1 or 3 days, respectively. Various sublines of L929 cells differ in sensitivity to virus and interferon. Two different sublines--L929A and L929B--were screened in the assay. A subline from E.C. Borden, University of Wisconsin, was chosen for use. This line (L929B) formed better monolayers in the plastic trays and proved to be more sensitive (more cells are protected) to a standard interferon sample at all challenge virus concentrations tested (Table 33). For example,

Table 32. Cell Concentration-Effect of Interferon Titer.

Seeding Cell Concentration* (cells/ml)	IF Titer**
2.0×10^5	1500
1.5×10^5	2100
1.0×10^5	1800
0.5×10^5	1900

*0.75 ml of cells were inoculated into each well. At 24 hours, a standard interferon was used to protect against VSV. Subsequently the titer of IF was determined.

**Reciprocal dilution of interferon

Table 33. Interferon Standard (IF 15-77) on L929A and L929B Cells with VSVB and VSVA as Challenge Virus*

Amount of Challenge Virus (pfu/well)	Interferon Titer			
	VSV B as Challenge Virus on:		VSV A as Challenge Virus on:	
	L929A Cells	L929B Cells	L929A Cells	L929B Cells
2.5×10^7			<<400	<<400
3.5×10^6	<<400	<<400		
2.5×10^6			<<400	<<400
3.5×10^5	<<400	2300		
2.5×10^5			<400	2300
3.5×10^4	850	2900		
2.5×10^4			1200	2300
3.5×10^3	1000	2600		
3.5×10^2	----	2200		
3.5×10		2900		

*Serial two-fold dilutions (1:400 to 1:3200) of interferon standard IF 15-77 were tested for protection of either L929A or L929B cells against viruses VSV A and VSV B. Interferon titer is the reciprocal of the interferon dilution which protected 50% of the cells at that viral concentration.

using Vesicular Stomatis Virus (VSV B) at $3.5x10^4$ pfu/well as the viral challenge, the IF sample titered 1:850 on L929 A cells and 1:2900 on L929 B cells.

c. Interferon

Interferon samples are prepared as below. Generally, two-fold dilutions are used for precision and accuracy in the assay. Cells are treated with the interferon samples for 24 hours but by 4 hours antiviral effect is evident. The in-house standard preparation of interferon (IF 15-77) titers 1:3075 with a standard deviation of 1790 (22 determinations). NIH reference standard G002-904-511 titers 1:5200 (1 determination). Determinations from 7 laboratories using a VSV plaque reduction assay resulted in a mean of 1:6485 (standard deviation of 5416) with this NIH reference standard. Although there can be a wide variation in the absolute titers of interferon samples from 1 assay to another (reproducibility), the relative titers of samples within 1 assay is maintained in other assays of the same samples (Table 34).

d. Virus

VSV, although only moderately sensitive to interferon, is the most commonly used challenge virus in interferon assays. Some arboviruses such as SFV, Sindbis and Chikungunya have been reported as being more sensitive in IF assays and thus have been used for challenge virus. In this laboratory Sindbis virus showed little CPE at 2 days on the L929 cells and therefore was not used. The risk of possible human infection contraindicates the use of Chikungunya as the viral challenge. SFV, both the avirulent A774 strain and the lethal L10 strain, proved to be both sensitive to interferon and cytotoxic for L929 cells. However, SFV was used in some experiments as an inducer of interferon and therefore to avoid any possibilities of SFV-specific, non-interferon inhibitors or viral growth in the IF assay, SFV could not be used as the viral challenge.

Two different strains of VSV, New Jersey, were tested on L929 B cells. There was little difference in the sensitivities of these strains to the interferon standards (Table 33). The interferon titered 1:2300 on L929 B cells when challenged with $3.5x10^5$ pfu/well of VSV B or with $2.5x10^5$ pfu/well of VSV A. VSVB, however, usually caused more cytopathic effect in cells

Table 34. Intra-Assay Relative IF Titers - Comparison Between Two Assays

Sample*	Interferon Titer	
	Assay 1	Assay 2
1	500**	<800
2	2700	1300
3	2550	1250
4	7500	3800

*The same interferon samples were assayed at 2 different times.

**Reciprocal interferon dilutions

Table 35. Cytopathic Effect Measured by Optical Density on Viral Controls

Challenge Virus Added (pfu/well)	VSV A	VSV B
2.5×10^7	.132*	
3.5×10^6		.130
2.5×10^6	.154	
3.5×10^5		.075
2.5×10^5	.213	
3.5×10^4		.142
2.5×10^4	.172	

*Optical Density is the average OD of at least 3 wells of cells. Variation of OD between identical wells is <10%.

not treated with interferon (viral controls) than did VSVA (Table 35). For this reason, VSB is used as the challenge virus. The effect of MOI of challenge virus on the titer of interferon sample was examined; at higher multiplicities of viral challenge (>0.5) interferon titer decreased (Table 36), whereas, with MOI's between approximately 0.1 and .001 interferon titer tended not to vary significantly. Low multiplicities necessitated extending the duration of incubation of the challenge virus for cytotoxicity to become evident.

Table 36. Effect of Challenge Virus MOI on Interferon Titer of Interferon Standard IF 15-77 on L929B Cells.

Amount of Challenge VSB B (pfu/well)	Approximate MOI	Interferon* Titer
$3.5x10^5$	1.0	1375*
$1.7x10^5$	0.5	2400
$3.5x10^4$	0.1	3600
$1.7x10^4$	0.05	4000
$3.5x10^3$	0.01	3750
$3.5x10^2$	0.001	5200
3.5x10	0.0001	>6400

*Reciprocal of the dilution that protects 50% of cells.

2. Interferon Inductions

In this laboratory, in vitro induction of interferon production generally is done in 6-well trays (Linbro, 5 ml/well). For mouse interferon, wells are inoculated with 2 ml of $2x10^5$/ml of L929 B cells and are ready for induction in 1-2 days. Growth media is aspirated, the inducer (1.0 ml) is added to the center of the monolayers by means of an automatic pipette and the tray is gently rocked. Different inducers are always inoculated into at least duplicate wells. After 1 hour at 37°C, the inducer is aspirated, the cells washed once with 1 ml EBSS, and 2 ml growth media added. Unless

the kinetics of interferon are being studied, the growth media containing the interferon is harvested at 24 hours. Samples induced by polyriboinosinic: Polyribocytidylic acid (rIn:rCn) need no further treatment and can be stored indefinitely at -70°C. Samples induced by virus are treated with 0.15 M perchloric acid for 30-90 minutes, and the supernatant containing the interferon dialyzed against EBSS to restore the pH to 7.3. These samples are then diluted 1:1 with HBSS-199 with 2% FCS and stored at -70°C. Virus-induced interferon preparations are tested undiluted at 10^{-2} dilutions for the presence of remaining virus. No virus has been demonstrated; thus the antiviral effect is not secondary to non-interferon viral interference.

L929 cells, unlike rabbit or human cells, produce little or no interferon in response to rIn:rCn alone. The rIn:rCn must first be complexed with DEAE-dextran, presumably resulting in enhanced adsorption and protection from nucleases. To determine the optimal amount of rIn:rCn/DEAE-dextran for inducing interferon production, a dose response curve was done (Table 37). Neither rIn:rCn nor DEAE-dextran alone produced interferon. With a constant concentration of rIn:rCn, production of interferon correlated with higher concentrations of DEAE-dextran. A ratio of 1:4 rIn:rCn/DEAE-dextran results in optimal interferon production as previously reported. As a result of these findings, interferon inductions on L929 B cells are generally done with rIn:rCn/DEAE-dextran at 100 ug/ml:400 ug/ml. In-house interferon standards have been made by inducing several 75 cm^2 flasks of L929 cells with Newcastle's Disease Virus (NDV) or rIn:rCn/dextran.

For in vivo inductions of interferon, rIn:rCn (25 ug/mouse) is injected IV and the interferon harvested at various times. Viral in vivo inductions are done IP. These interferon samples are prepared as above.

B. Differences in Interferon Induction and Sensitivity Between Two Strains of Semliki Forest Virus (SFV): A774 and L10 Viruses.

The A774 strain of SFV is avirulent in several mouse lines whereas the L10 strain is highly lethal. With the exception of higher virus titers and prolonged presence of the L10 strain in brain and spleen, little can be distinguished between the infections of these two viruses in mice.

Table 37. Dose Response of rIn:rCn ± DEAE-Dextran and Induction of IF Production*

rIn:rCn** (ug/ml)	DEAE-dextran** (ug/ml)	Interferon Produced***
50	0	0
100	0	0
200	0	0
100	50	14
100	100	700
100	200	1800
100	400	4000
100	800	3600
200	100	<800
200	200	1300
200	400	1250
200	800	3800

*L929 B cells.

**When complexed together the rIn:rCn and DEAE-dextran were mixed at room temperature for 15-30 minutes prior to induction. All concentrations are final after mixing. rIn:rCn was obtained from Gibco. All dilutions were made in EBSS.

***Reciprocal of dilution protecting 50% of cells.

Efforts to explain the virulence differences on the basis of defective interfering particles or interferon have been unsuccessful. In relatively insensitive interferon tests, interferon was found in brain and blood in low amounts and was not felt to be related to the primary expression of virulence. In further characterization of the differences of A774 and L10 infections, inoculation of mice with avirulent A774 protected mice from the lethal L10 in a biphasic manner. Late protection (after 6 days) correlates well with the presence of specific serum antibody. An early protective phase

(18 hours to 4 days) is nonspecific, can be ablated with silica and cytoxan, and is not associated with antibody. Efforts were begun to evaluate the possible role of interferon in the pathogenesis of L10 and A774 infections and the early protective phase induced by A774. Initial work emphasized the capabilities of A774 and L10 to induce interferon *in vitro* and respond to it.

Duplicate wells of L929B cells were infected with L10 and A774 viruses at several different MOI's. At 24 hours, the supernatant was harvested and interferon assayed. No interferon was detected in the supernatant from cells induced by the avirulent A774 (Table 38), whereas the induction of interferon by L10 correlated with the MOI. The low yield of interferon at low MOI conceivably resulted from harvesting the supernatant too early; thus, the kinetics of interferon production were examined. At 48 hours, interferon was produced in response to A774 but still in lower amounts than that produced by L10.

Table 38. Differential Induction of Interferon by L10 and A774 Strains of Semliki Forest Virus.

Virus Strain	MOI	Interferon* Titer
L10	0.01	65
	0.1	310
	1.0	780
	10	1150
A774	0.01	0
	0.1	0
	1.0	0
	10	0

*1/dilution resulting in 50% protection.

Duplicate wells of L929B cells were infected with L10 and A774 viruses at several different MOI's. At 24 hours, the supernatant was harvested and interferon assayed. No interferon was detected in the supernatant from cells induced by the avirulent A774 (Table 38), whereas the induction of interferon by L10 correlated with the MOI. The low yield of interferon at low MOI conceivably resulted from harvesting the supernatant too early; thus, the kinetics of interferon production were examined. At 48 hours, interferon was produced in response to A774 but still in lower amounts than that produced by L10.

Since temperature sensitive mutants of SFV can vary in their ability to induce interferon depending on temperature and MOI, (Lomniczi and Burke, 1970), A774 and L10 were tested at 2 different MOI's for the induction of interferon at 30°C, 35°C, and 39°C. A774, in this experiment, did not induce interferon production at either high or low MOI's at any of the three temperatures (Table 39). As compared to other

Table 39. Induction of Interferon by L10 and A774 at Various Temperatures.

Virus Strain	MOI	Interferon Titer at 24 Hours*		
		30°	35°	39°
L10	.01	<20	<20	45
	1.0	23	215	350
A774	.01	<20	<20	<20
	1.0	<20	<20	<20

*Reciprocal serum dilution

experiments, L10 induced less interferon than usual, but it still produced significant amounts at a MOI of 1.0. Higher temperatures were associated with greater interferon production. It is possible that A774 would produce detectable interferon in cultures held longer than 24 hours. Nonetheless, the data suggest that lack of interferon induction at 37°C by A774 is not a result of a temperature sensitive step which would be reversed at 30°C.

Correlations between high interferon production and both good and poor virus growth have been made. Likewise, low interferon production has been associated with both good and poor virus growth. Finter (1964) described two strains of SFV, one of which grew to lower titers, yet produced more interferon. No studies of virulence of these two strains were done. To examine both the growth characteristics and interferon-inducing capabilities of A774 and L10, L929B cells in 6 well trays were challenged at 2 MOI's. At each time point, 2 ml of media was removed from duplicate wells and the wells refed; these samples were assayed for interferon. In a duplicate set of wells, 0.2 ml of media was removed for virus plaque assay and replaced with fresh media. In this experiment, A774 induced some interferon, but in significantly lower amounts than L10 (Tables 40 & 41). Peak interferon production was detected at 48 hours when challenge virus was at a MOI OF 0.01 and at 24 hours for a MOI of 10. L10 produced more interferon than A774, yet the latter virus grew to a higher titer. L10 virus reached peak titers at 24 hours, whereas A774 peaked at 48 hours. Simplistically, it appears that the higher induction of interferon by L10 results in early suppression of viral growth.

Preliminary results indicate that L10 might also be more sensitive to interferon than is A774. Monolayers of L929B cells were overlayed for 24 hours with dilutions of a standard interferon sample (IF 15-77). At 24 hours, interferon was removed and 40-60 pfu of either L10 or A774 was adsorbed to the cells. After 1 hour at 37° the cells were overlayed with agar. Plaques developed after 48 hours. A 1:4100 dilution of interferon reduced the number of L10 plaques to 50% of control, but it took a 1:2800 dilution of interferon to reduce the A774 plaques to 50% of control.

Studies are underway to determine if the differences in interferon induction and sensitivity between SFV strain

Table 40. Interferon Titers at Different Times after Infection by two strains of Semliki Forest Virus.

Virus Strain	MOI	Interferon Titers at Hours Post Inoculation 6	24	48	72	96
L10	.01	<10*	640	2000	60	10
	10	<10	980	100	20	10
A774	.01	<10	180	280	<100	10
	10	<10	310	<100	25	10

*Reciprocal serum dilution

Table 41. Virus Titers at Different Times after Infection by Two Strains of Semliki Forest Virus

Virus Strain	MOI	Viral Titers at Hours Post Inoculation 6	24	48	72	96
L10	.01	1.5×10^2	5.7×10^5	5.2×10^5	2.9×10^5	1.0×10^5
	10	7.9×10^4	2.9×10^6	8.5×10^5	4.5×10^5	3.9×10^5
A774	.01	5.0×10^2	1.0×10^5	2.5×10^7	9.5×10^5	7.5×10^5
	10	2.4×10^5	4.3×10^6	1.7×10^7	2.8×10^6	1.9×10^6

L10 and A774 observed *in vitro* also occur *in vivo*. On the basis of experimental results available so far, the differences in virulence between L10 and A774 cannot be explained by interferon production alone.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 130, Viral Infections of Man

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6443	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	132
b. CONTRIBUTING				
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11. TITLE (Precede with Security Classification Code)

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19. RESPONSIBLE DOD ORGANIZATION

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pseudomonas aeruginosa; (U) Neisseria meningitidis; (U) Gonococcus; (U) Immunology; (U) Antibiotics; (U) Infectious Diseases

23. TECHNICAL OBJECTIVE; 24. APPROACH; 25. PROGRESS (Furnish SAME for each program element listed in Block 10. Precede EACH with Security Classification Code)

23 (U) Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on control of meningococcal, gonococcal, pseudomonas and staphylococcal infections in military forces.

24 (U) Development of bacteriologic techniques for study of various infectious diseases. Field studies on prophylactic regimens, spread and persistence of organisms in various military populations.

25 (U) 77 10-78 09 A group B meningococcal vaccine consisting of high molecular weight group B polysaccharide complexed with outer membrane protein has been developed and shown to be safe and to produce bacteriocidal antibodies in volunteers. Meningococcal polysaccharide vaccines against groups Y, W135 and 29E have been shown to be safe and immunogenic in volunteers. A high molecular weight polysaccharide has been isolated from Pseudomonas aeruginosa and this protects mice from experimental infection Genital antibodies to gonococcal pili and native cell wall complex are able to block attachment of gonococci to human epithelial cells. Of the mononuclear cells in the blood, only killer lymphocytes or monocytes kill bacteria in the presence of specific antibody. IgA and IgG are equally effective in this system, while IgM is relatively ineffective. With normal human sera antibody-mediated cellular cytotoxicity was more prevalent than complement-mediated killing. The K-1 antigen has been shown to be responsible for the serum resistance of rough gram negative organisms isolated from cases of human bacteremia. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 132 Bacterial Diseases of Military Importance

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1. A simple, reliable, and economical method for the detection of penicillinase-producing Neisseria gonorrhoeae using a penicillin disk and a penicillin-sensitive organism was developed.

2. Infecting gonococcal strains from 2 consorts isolated at different times were found to possess different attachment antigens. This antigenic variation may account, in part, for recurrent gonococcal infections.

3. A new transport media for GC has been developed.

4. Gonococcal pili and native cell wall complex competitively inhibit the attachment of gonococci to human buccal epithelial cells. Gonococcal lipopolysaccharide inhibits the attachment in a nonspecific manner. Gonococcal pili and native complex, but not lipopolysaccharide can adsorb the antibodies in vaginal secretions which inhibit the attachment of N. gonorrhoeae to epithelial cells.

5. Twenty-four thiosemicarbazone compounds, a class of drugs with antimalarial activity, were tested for ability to inhibit the growth of S. aureus, Group D. enterococcus, P. aeruginosa, K. pneumonia, S. flexneri, S. dysenteriae, E. coli, P. vulgaris, N. gonorrhoeae and N. meningitidis. Eight of these compounds were found to have promising activity.

6. A pilot-plant batch of a nontoxic polysaccharide has been prepared for Pseudmonas aeruginosa, type 1.

7. An immunochemical relationship between diphtheria toxin and P. aeruginosa Toxin A has been established.

8. The use of rough core-defective mutants of Salmonella minnesota has given some insight into the cell receptor sites of pyocins.

9. An improved method for preparing streptococcal group B polysaccharide has been formulated.

10. The antibody response of patients with bacteremia at WRGH has been studied.

11. Rough strains of E. coli which possess the K1 antigen are resistant to the bactericidal action of serum.

12. A factor released from PHA-activated mononuclear cells which enhances the bactericidal activity of granulocytes has now been shown to be produced by long-term cultured lymphocytes.

13. A high proportion of organisms isolated from the blood of patients at WRGH have been shown to carry transferable drug resistance.

14. A vaccine against meningococcal group B disease has been developed and found to be safe and immunogenic in volunteers.

15. Antibodies against the meningococcal outer membrane proteins which arise after natural infection are directed against labile determinants.

16. A continuous in-line chromatographic process for the separation of IgA, IgM and Ig in small volumes of serum has been developed.

17. An outbreak of meningococcal group A disease in the Pacific Northwest has been studied.

18. The immune response of a pediatric population following infection with meningococci has been studied.

19. Capsular polysaccharide vaccines against meningococcal minor serogroups Y, W135, and 29E have been prepared and preliminary tests for safety and immunogenicity have been conducted in volunteers.

20. Purified IgA from sera of patients convalescing from group C meningococcal disease can induce monocyte-mediated bactericidal

activity.

21. In vitro cultured methods have been developed to measure the ability of human peripheral blood lymphocytes to produce IgG, IgM or IgA.

22. Antiserum against type 14 pneumococci exhibits opsonic activity against group B type III streptococci.

1. Small, low volume field laboratories, assigned to military field units require a simple test for detecting penicillinase-producing Neisseria gonorrhoeae for which test materials and control organisms can be easily stored and maintained. The following procedure has been devised:

A penicillin-sensitive organism, preferably Staphylococcus aureus, is streaked upon a plate in a manner similar to that used for antibiotic sensitivity testing by the Bauer-Kirby methods. A penicillin disk (10 U) is placed on the lawn of organisms. A heavy inoculum of test organism is streaked outward from the disk. Strains producing penicillinase distorted the zone of inhibition. A penicillinase-producing (not necessarily gonococci) and non-penicillinase-producing organisms are also streaked as controls. Penicillinase-producing gonococci, as well as other penicillinase-producing organisms, such as Haemophilus influenzae, Escherichia coli, Serratia marcescens, and S. aureus, can be detected. Furthermore, the amount of penicillinase produced by the unknown organisms can also be quantitated. This simple technique has the advantage of utilizing a standard antibiotic sensitivity testing method and readily available penicillin sensitivity discs. Maintaining penicillin sensitive staphylococci on an agar slant or a plate over a long period of time is not difficult. Preliminary results can be obtained in 6 hr., and this technique is sensitive and specific for penicillinase production. Also, non-betalactamase-mediated resistance can be discerned when an unknown organism grows in the presence of penicillin, but does not distort the zone of inhibition of the test organism.

2. Based on bactericidal typing, pyocin typing (table 1) and antibiotic sensitivity tests, 2 consorts appear to have been reinfected 34 and 41 days later, respectively, with the same gonococcal strain, suggesting a failure in these patients to develop effective immunity to re-infection. However, these tests did not measure antigens which mediate attachment, a function which may correlate with virulence. When the above infecting strains were tested in an inhibition of attachment

assay, using rabbit gonococcal antisera, the antigens mediating attachment were found to be different (table 2). Homologous antisera inhibited attachment of the homologous strain at a high titer. Absorbing the antisera with the initial infecting strain did not remove any of the blocking activity of the antisera raised to the re-infecting strain, but the latter strain did share some attachment antigens in common with the initial infecting strain. Homologous antisera also bound preferentially to pili purified from the homologous strains (table 3).

3. Neisseria gonorrhoeae do not survive transport by mail very well. In order to better define the epidemiology of gonorrhea in certain geographical areas (i.e., Ft. Bragg, Korea) an improved transport medium was developed, so that strains could be sent to WRAIR, for study. This medium is basically a modification of other previously described formulae. After overnight incubation at 37° under five to ten percent carbon dioxide, gonococci remains viable at room temperature for up to 7 days. This medium is currently being tested under field conditions in collaboration with personnel based at Ft. Bragg. The formula for the medium is:

Transport Medium

1. Protease Peptone #3 (Difco)	15 gm.
NaCl	5 "
Dipotassium phosphate ($K_2H\ PO_4$)	4 "
Potassium (KH_2PO_4) dihydrogenphosphate	1 "
Soluble Starch	1 "
$NaHCO_3$	150 mg.
$D.H_2O$	790 ml.
pH 7.2 - adjust	
2. Add Oxoid Agar	9 gm.
Bring to boil	
Autoclave 15 min.	cool
3. Add 100 ml. inactivated Horse Serum (56° - 30 min)	
4. Add Dextrose	5 gm. in 100 ml. $D.H_2O$
5. Add 20 ml. Defined Supplement 100 ml.	

Sol. I 80 ml. 50% Dextrose
1 ml. 0.2% cocarboxylase
Sol. II 83 mg $Fe(NO_3)_3 .9H_2O$
in 19 ml. $D.H_2O$

1 gm. L-glutamine
Mix two solutions, filter and freeze

6. Add 10 m. VCN Inhibition
Vancomycin 3 mcg/ml.
Colistan 7.5 mcg 1 ml.
Nystatin 12.5 units/ml

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4. Gonococcal pili, lipopolysaccharide (LPS), and native complex (NC), were studied to determine which antigen is primarily responsible for inducing the attachment-inhibiting antibody. Gonococcal pili were purified using the method of Brinton. When the pili preparation was examined, using SDS polyacrylamide gel electrophoresis, only a single protein band was present. Ultraviolet spectrophotometry confirmed the absence of contaminating nucleic acid in the preparation. Lipopolysaccharide was extracted, using the phenol-water method of Westphal, and further purified using a G-100 Sephadex column. Native cell wall complex was prepared, according to the method of Zollinger. This preparation was shown to contain pili by its ability to inhibit the binding of antigonococcal rabbit antibody to purified pili antigen in the solid phase radioimmunoassay. The native cell wall complex of colony type T2 used throughout this study, was shown to contain LPS by determining the 2 keto-3 deoxy (KDO) sugar content of the sample by Osborne assay. Again, absence of nucleic acid was confirmed using UV spectrophotometry. Pili, LPS or NC were used as the antigen in the solid phase radioimmunoassay (SPRIA). Radio-labelled antihuman, goat antisera was used as the secondary antibody. Genital secretions from a single infected patient were collected over a four month period, pooled and concentrated to 25 µg/ml total antibody as determined by SPRIA. The patient was treated when the diagnosis was first made. Inhibition of epithelial cell adhesion was determined as previously described (Annual Report, 1975). Because of the competitive inhibition of attachment of gonococci by the antigens used in the assay, an inhibition test could not be done and the vaginal secretions had to be absorbed to remove attachment-inhibiting antibody. The absorptions were carried out by mixing pilus crystals, NC or, LPS bound to alum, with the vaginal secretion. The antigen complexes were removed by

centrifugation. The efficacy of the absorption of gonococcal antibody was determined using the solid phase radioimmunoassay in which the amount of antibody in the absorbed antisera that bound to a specific gonococcal antigen, was shown to be reduced when compared to the amount of antigen-binding antibody, in unabsorbed antisera.

It was found that local antibody, inhibiting attachment of gonococci to epithelial cells was removed by the homologous pili and native complex. On the other hand, gonococcal LPS was incapable of removing inhibiting antibody (table 4,5).

5. The thiosemicarbazones have been studied as potential agents for the treatment of mycobacterial poxvirus, and malaria infections. As a preliminary screening, 24 compounds previously screened by Division of Experimental Therapeutics in their antimalarial drug program, were tested for minimal inhibitory concentration against a wide variety of bacteria (table 6). In general, the spectrum paralleled that seen with penicillin, especially for those compounds showing the least toxicity in mice, i.e., compounds H & L. The activity is unaffected by penicillinase.

6. Polysaccharides were isolated from each of the seven Fisher-Devlin immunotypes of Pseudomonas aeruginosa. Each was shown to be of high molecular weight, serologically related to lipopolysaccharide, and protective in mouse challenge experiments. Cross protection was elicited between types 1 and 2, when 50 µgm doses of polysaccharides, were used for vaccination of the animals. Although immunotype specific protection could be elicited by 10-20 nonogram doses of lipopolysaccharide vaccines, cross protection was not elicited until 25-50 µgm vaccine doses were employed. The LPS vaccines, but not the polysaccharide preparations, were toxic for mice at these doses. This suggests that the high molecular weight polysaccharides derived from P. aeruginosa may be tolerated by humans in high enough doses to elicit cross protection among serotypes.

A thirty liter batch of P. aeruginosa immunotype 1, was grown in Trypticase Soy Broth at the Forest Glen Facility. See 1978 Annual Report, Department of Biologic Research, Work Unit 136. Cetavalon (0.1 percent, final concentration) was added and phenol 0.5 percent final concentration, was added. The pellet containing organisms and nucleic acid-Cetavalon complexes was removed by continuous flow centrifugation and the supernatant passed through 0.45A millipore filters. The supernatant fluid was then concentrated on a PM-30 amicon filter to 600 ml. and

was precipitated with 400 ml ethanol. The pellet was redissolved in PBS. Cetavalon was added to a final concentration of 0.1 percent, the precipitate collected, and discarded. The supernatant was precipitated with 4 volumes of alcohol. The pellet was collected, and dissolved in 400 ml of 1 percent acetic acid, and maintained at 90°C for 18 hours. This was the first step in a procedure to eliminate LPS by cleaving the acid labile link between lipid A and 2 keto deoxyoctonate (KDO) in the LPS molecule. The suspension was cooled, and the precipitate removed. Three successive chloroform extractions were performed, to remove contaminating lipid. The material was alcohol precipitated, washed in alcohol and acetone, redissolved in PBS, and treated with an equal volume (100ml) of cold 90 percent phenol. After collection of the water layers, this step was repeated. The water layers were precipitated with alcohol. The precipitate was redissolved in PBS, and pressed over 7.5 liter bed volume columns of Sephadex G100. A large peak was identified in the void of anthrone and phenol sulfuric reactions for carbohydrate. This peak was pooled, alcohol precipitated, and redissolved in 150 ml of water. The product was ultracentrifuged at 100,000 x g for 1 hour, the supernatant collected, and precipitated with alcohol, redissolved in water, and dialyzed for 72 hours, against distilled water. The concentration was adjusted to 1 mg/ml, filter sterilized and frozen. Sterility tests were performed, and the vaccine was shown to be sterile. The vaccine was bottled, and sampled for sterility. The vaccine was non-pyrogenic in rabbit testing at the 0.25 µgm level, which is 100 times better than the current meningococcal standard. Analysis for LPS, by limulus lysate, did show a ∿2 percent LPS contamination, by that method. Protein could not be detected by the method of Lowry. By amino analysis, that vaccine contained 1 percent protein. Analysis revealed that per 1000 µgm of pseudomonas vaccine 884.3 µgm of neutral and acidic sugars, 115.6 µgm of amino sugars, amino acids, and unknown material. Serum from patients infected with Pseudomonas aeruginosa have been anlyzed for opsonic antibody. To date, lipopolysaccharide, and outer membrane complex antigens, are the most efficient at absorbing opsonic antibody found during the course of human infection. Purified polysaccharides, and polysaccharides isolated from LPS, have not efficiently absorbed opsonic antibody. For example, patient U, had continuous Pseudomonas aeruginosa bacteremia for 4 months, secondary to tricuspid valve endocarditis. His opsonic titre in the microphagocytic assay was 1:128 (final dilution 1:1280). His organism was an immunotype 6 by agglutination, and solid phase immunotyping.

His sera was tested in the microphagocytic assay at a dilution of 1:10 (final dilution 1:100). Type 6 polysaccharide was unable to inhibit the reaction. The uninhibited sera, plus polymorphonuclear white blood cells killed 85 percent of the organisms as calculated by comparing the number of viable organisms in the reaction mixture at 90 minutes compared to the initial numbers at the beginning of the experiment (zero time). Addition of LPS prepared by phenol water extraction from patient U's organism at a final concentration in the test of 50 µgm/ml reduced the killing at 90 minutes to 29 per cent of the initial inoculum. In other words, LPS was able to inhibit 66 percent of the antibody mediated opsonic kill of this organism. Final concentration of 5 µgm LPS/ml inhibited 34 percent of the kill, and .5 µgm LPS/ml was unable to inhibit any of the kill with 85 percent of the organisms being killed after 90 minutes - a value identical to the uninhibited control value. The inhibition was not due to non-specific anti-complementary effects since 50 µgm LPS/ml inhibited 100 percent of the opsonic antibody mediated kill in a complement independent system. We have found that high titred sera such as that in patient U, gives the same degree of opsonic mediated killing with or without the addition of exogenous complement. At a dilution of 1:10 patient, U's serum killed 81 percent of initial inoculum without exogenous complement. At a dilution of 1:100 (final dilution 1:1000) without exogenous complement patient U's heated sera killed 99.4 percent of the organisms. Interestingly, the more dilute sera have consistently been able to kill a larger number of organisms, implying some role for blocking antibody factors at the lower dilutions. This is not inconsistent with serum killing in the bactericidal reaction seen in the chronic infections such as brucellosis. Attempts were made to purify polysaccharide from the LPS utilizing acetic acid. The material could be recovered in low yield, and was unable to inhibit the opsonic mediated complement dependent killing. This is consistent with other data on the type 6 lipopolysaccharide which suggests that the important antigenic determinants in its lipopolysaccharide are acid labile. In summary, analysis of patient's sera has yet to demonstrate that opsonic antibody is directed against the acid stable component of the lipopolysaccharide molecule, but is directed against determinants in the LPS molecule. A limited number of sera have been looked at, and more must be examined before this conclusion is warranted. One particular patient's sera has been especially interesting to analyze because this patient developed the kind of high titred opsonic mediated protection we hope to achieve through vaccination.

7. Studies of the human antibody response to Pseudomonas Toxin A, during and following infection with Pseudomonas aeruginosa were continued. The findings indicated that, patients who died of pseudomonas sepsis at Walter Reed Hospital did not produce antibody to Toxin A, (11/12 patients). Three of the patients, however, were infected with strains of P. aeruginosa that did not produce Toxin A, but rather the newly discovered Exoenzyme S. Several of the patients died rapidly, before any antibody response could have been expected. The findings suggest that Toxin A, and possibly Exoenzyme S, may be playing a role in clinical P. aeruginosa infection. The lack of response to Toxin A, in patients who subsequently died of pseudomonas sepsis, may only reflect a general inability to respond to antigenic stimuli, and may be a reflection of the severity of their illnesses. In four of the patients examined thus far, opsonic antibody could be demonstrated despite the lack of anti-Toxin A antibody. This would be evidence against the proposition that the patients who died had a general inability to respond. Likewise, three patients who survived Pseudomonas aeruginosa sepsis, and made good antitoxin A antibody shortly thereafter, died of gram negative sepsis due to other causes, and again suggestive that antitoxin A antibody may play some mediating role in protection.

Although pseudomonas Toxin A, and diptheria toxin, have the same molecular mechanisms of action, they have not been shown to be related antigenically or chemically. In fact, all available evidence would indicate that they are not related immunologically, in that antitoxin A, neither inhibits ADP ribosylation by diphtheria toxin or fragment A of diphtheria toxin, nor protects in tissue-culture toxicity or animal model systems. Likewise, diphtheria antitoxin does not neutralize the enzymatic activity of Toxin A, or neutralize its tissue culture or mouse toxicity. A series of solid phase radioimmunoassay inhibition experiments were performed to examine the immunochemical relation between these two toxins in more depth. Small, but measureable direct binding was demonstrated between Exotoxin A, as antigen, and (1) rabbit anti-diphtheria toxoid (2) anti-fragment A of diphtheria toxin (3) anti-B fragment of diphtheria toxin (very small amount of binding). Likewise, a small amount of binding, above background, could be demonstrated by anti-pseudomonas toxin A for intact diphtheria toxin. Solid phase inhibition studies were then performed.

The data show that only trypsin-treated diphtheria toxin, and Fragment A of diphtheria toxin were able to inhibit the reaction between pseudomonas anti toxin-A, and toxin A (table 7). This indicates that the, heretofore, undetected, common antigenic determinant between these two toxins is found in the diphtheria toxin molecule and is located somewhere on the A fragment. Since the inhibition with trypsin-treated diphtheria toxin is not complete, it would suggest that the shared determinant is an immunochemical one for pseudomonas toxin A. Likewise, the fact that pseudomonas toxin A, cannot inhibit the diphtheria-anti-diphtheria toxin reaction, suggests that the common determinant between toxin A and diphtheria toxin is not the immunodominant diphtheria toxin determinant. These immunochemical relations, coupled with common NAD dependence and glycohydrolase activities for pseudomonas toxin A, diphtheria toxin and cholera toxin suggest the possibility of a common ancestral protein toxin for these three bacterial virulence factors.

8. Pyocins, induced by mitomycin C from P. aeruginosa, and purified by ultracentrifugation, were shown capable of killing core-defective R-mutants of Salmonella minnesota in a selective manner which shed light on the chemical nature of the receptor in the lipopolysaccharide core (table 8). The smooth strains with complete O side chains were resistant to all pyocins tested as were the Re mutant, containing lipid A and KDO and the Rd_2 mutant containing lipid A, KDO and a single heptose in their LPS. the Rd_1P^- mutant identical to Rd_2, except for the addition of a second heptose to the LPS structure, was sensitive to pyocins D, F, G, H, I. This indicated that the heptose-heptose area may be the receptor area for these pyocins. The Rd_1P^+ mutant differs from the Rd_1P^- mutant, by the addition of pyrophosphorylethanolamine. This group appears to have the ability to block receptor activity as this mutant was resistant to pyocins D, F, G, H, and I. Mutant R_cP^- differs from the Rd_1P^- by the addition of glucose to the second heptose and containing no pyrophosphorylethanolamine. This mutant R_cP^- was also resistant to pyocins D, F, G, H, and I, indicating that the heptose receptor region was not available. Mutant strain R_cP^- was sensitive to Pyocins A, B, C, E, G, K, M, N, O, P, R, S, and T suggesting all of these pyocins have receptors related to the terminal glucose. Once again, the addition of a pyrophosphorylethanolamine group, plus an additional phosphate, and heptose to the second molecule, is found in mutant R_cP^+, blocked the ability of pyocins to kill the mutants. The Rb mutant is identical to the R_cP^+, plus the

addition of glucose-galactose and galactose to the terminal glucose of R_cP^+, thereby, creating a terminal glucose at some distance from the changed phosphate, and pyrophosphorylethanolamine groups. The Rb mutant was sensitive to the same pyocins as the R_cP^-, but the titres of pyocin activity were considerably lower for Rb as compared to R_cP^-. The Ra mutant differed from the Rb strain only by the terminal additional of N acetylglucosamine. Interestingly, this mutant is sensitive in low titres to the pyocin, whose receptor area appears to be in the heptose region (D, F, H, I & J), and to the other pyocins with activity toward the glucose region or, if in this slightly longer core, the molecule had opend up, somewhat.

The specificity of the reactions was confirmed by absorbing with LPS prepared by the Galanos method from each of these mutants. LPS from mutants that were killed by a particular pyocin were able to absorb activity, while LPS extracted from mutants that were not killed by a particular pyocin, could not absorb activity. Thus, pyocin D's activity could be absorbed by LPS extracted Rd_1P^- and Ru but not by LPS from Re, Rd, Pt or any other mutant LPS strains. When pyocin activity was absorbed with Salmonella core mutant LPS, it lost activity against (1) Salmonella core mutants (2) P. aeruginosa and (3) Neisseria gonorrhoeae. Likewise, LPS from N. gonorrhoeae and LPS from P. aeruginosa were both able to absorb pyocin activity for Salmonella core mutants as well as P. aeruginosa and N. gonorrhoeae, indicating that each of these organisms contain common heptose-heptose, and glucose receptor regions in their respective LPS structures.

9. Group B streptococci represents a serious cause of neonatal sepsis, and meningitis. In the course of biochemical investigations into methods of isolating gonococcal polysaccharide capsules, it became apparent that a new technique for the isolation of Group B streptococcus type III polysaccharide could be applied. Antibody against the Group B type III polysaccharide is thought to be opsonic and protective when passively transferred. Current techniques for isolation of this polsaccharide are cumbersome, and the yeilds are low - primarily because the polysaccharide must be isolated from the organism. The new technique makes use of the fact that the polysaccharide sticks to ion-exchange resins. The organisms are grown in Todd-Hewitt broth or in a media consisting of casein hydrolysate 3.1 g; proteose peptone #3 20.0 g; dextrose 4.0 g; sodium chloride 2.0 g; disodium phosphate 1.6 g; sodium carbonate 2.5 g per 1000 ml of distilled water.

The culture is centrifuged and the organisms discarded. The supernatant is then diluted with four volumes of distilled water to lower the ionic strength. Fifty grams of prewashed Whitman DEAE cellulose is then added and stirred for 1 hour. Then it is allowed to settle and the supernatant is discarded. The resin is collected and washed on a Buchner funnel with 0.01M Tris buffer (pH 7.5), and then eluted with 0.1M Tris and 0.25M Tris pH 7.5. The polysaccharide elutes at 0.25M. The wash is then ultrafiltered with 0.01M Tris in an Amicon PM-10 membrane and then pumped into a DEAE CL-6B sepharose (Pharmacia) column. Cultures labeled with C^{14} Na Acetate yielded two peaks with immunoprecipitin activity against Type III group B and Type 14 pneumococcus both being found in the second peak. This pooled peak is then placed over sepharose 4B column and elutes just following the void volume indicating it is around 400,000 daltons. The yields are in the range of 5 mg polysaccharide per liter of culture as compared to the published yields of 5 mg per 40 liters. The polysaccharide is currently being chemically analyzed. Immunologically, it reacts with antisera made against Group B type III Streptococcus and Pneumococcus type 14. Although there are some data to suggest that antibody against the cross reactive common core determinants may be opsonic and protective in rabbits the preponderance of evidence at the current time indicates that antibody against the distinctive sialic acid-containing side chain is of primary importance in the epidemiology and immunology of human infections. Therefore, the unique streptococcal Group B type III polysaccharide will probably be the vaccine used in women of the child bearing age.

10. Studies on patients with gram-negative bacteremia from WRGH have continued with the objective of either preventing these infections or of devising improved methods of treatment. Analysis of the antibody response of these patients is nearly completed. The response of most patients is predominantly to the smooth side chains of the lipopolysaccharide (LPS) of the infecting organism. The response was rapid and occurred two to five days after bacteremia. Little or no response was observed to the rough core determinants. All patients responded with rises in opsonic antibody which did not require complement for activity. Absorption of serum with LPS extracted from the infecting organism effectively removed the opsonic activity. On the other hand, absorption with LPS obtained from rough mutants (E. coli J5 or S. minnesota Re) failed to affect opsonic activity. Antisera to the J5 or Re strains had no opsonic action in the presence of monocytes or granulocytes.

However, the opsonic potency of the J5 antiserum was enhanced by the addition of fresh human serum. The factors in the fresh human serum which are responsible for this increased activity have not yet been identified.

11. Seventy consecutive strains of E. coli isolated from cases of bacteremia at WRGH were studied to determine whether or not they were rough or smooth and wehther or not they possessed the K-1 antigen. All K-1 positive strains irrespective of whether or not they were rough or smooth were serum resistant. Rough strains are considered to be serum-sensitive, but obviously the presence of the K-1 antigen protects them. Mutants of rough strains which lost the K antigens were serum sensitive.

12. It was previously reported that PHA-activated human mononuclear cells release into their supernatants a factor that can enhance the bactericidal activity of granulocytes. These studies have been extended to show that supernatants from long-term cultured lymphoid cell lines also cause enhanced bactericidal activity in PMN's. The enchanced bactericidal ability is seen when Group B streptococcus type III is the target organism, as well as with Serratia marcescens. The enhancing factor is active after heating to 56°C, but not 95°C, and can be diluted to a titer of 1:64 and still retain significant activity. The serum factor is not dialyzable.

13. During the last three years there has been an increasing incidence of resistance among gram-negative bacteria to commonly used antibiotics. The incidence of transferable antimicrobial resistance was determined in bacteria which were collected from the Clinical Microbiology Laboratory at WRGH between 1976 and 1978. Mating experiments were done with 150 strains of E. coli, Klebsiella penumoniae and Pseudomonas aeruginosa. Over 70 percent of ampicillin resistance was transferable; carbenicillin, tetracycline and sulfa resistance were transferred in order of decreasing frequency. Multiple plasmids were seen in the majority of strains. Tetracycline resistance ususally occurred on a single plasmid. Despite the increasing use of gentamicin, tobramycin and amikacin in WRGH the last 3 years, transferable resistance was seen infrequently.

14. Purified high molecular weight group B polysaccharide given alone as a vaccine has not been immunogenic in human beings. Because natural infections with group B meningococci result in only very small increases of hemagglutinating or primary binding

antibody to the group B polysaccharide, it was felt that B polysaccharide would not be a suitable vaccine even when optimally presented. This conclusion was underscored by the work of Kasper, et al., at WRAIR, which suggested that antibody to the B polysaccharide was not bactericidal to all B strains. Consequently, noncapsular antigens, in particular, the serotype protein antigens were investigated as possible vaccine candidates. Two studies were conducted at WRAIR, in which several preparations containing the serotype 2 protein antigen (P2), were found to be immunogenic in animals, but not in human beings. We now know that the poor immunogenicity of these preparations in man resulted from inactivation of important antigenic determinants during preparation of the vaccines, and that these serotype antigens in their native conformation are immunogenic to man. Primary binding antibodies, and bacterial antibodies against the serotype proteins have been demonstrated in human sera following natural infections.

Antibodies to the type P2 proteins, however, would be expected to provide protection only against type P2 strains. It was thus encouraging when in a previous vaccine study (lots 138I-0 and 138I-M1), evidence was obtained that a vaccine containing outer membrane protein prepared from a group C, type P2 strain, could induce increases in bactericidal antibodies against the group B polysaccharide. It was subsequently found that 90 percent of adult normal human sera had bactericidal antibodies against the group B polysaccharide (4) and that contrary to the findings of Kasper, et al. (J. Inf. Dis. 127: 387, 1973), these antibodies were bactericidal for all group B strains tested. The difference between our results and those of Kasper, et al., is probably due to the fact that the anti-group B polysaccharide antibodies are now known to be nearly all of the IgM class, to lose activity as a result of repeated freezing and thawing or exposure of diluted serum to warm temperatures, to be directed against a labile determinant associated only with high molecular weight polysaccharides, and to exhibit variations in bactericidal activity with different lots of complement. These properties were not known or controlled for at the time Kasper did his work. These findings led to preparation, and testing of a new vaccine which consists of high molecular weight group B polysaccharide non-covalently complexed to antigenically active type P2 outer membrane protein.

Phase I testing in human subjects

A total of eight volunteers were pre-selected from a group of 22, for lack of colonization with meningococci, and for having low antibody titers, and vaccinated with one of two very similar vaccine preparations: BP2-WZ-2 and BP2-PA-3. These volunteers were given two 120 µg (dry weight) doses, five weeks apart. The vaccine was given subcutaneously in the deltoid region, and was preceded by a 1 µg intradermal skin test given in the volar region of the forearm 20-30 minutes prior to the vaccinations. Throat cultures, serum samples, and stool samples were obtained at 0, 2, 5, 7 and 10 weeks. Side effects of the vaccines consisted principally of mild local reactions at the sites of the skin tests and vaccinations in all volunteers. The reactions first appeared at 2-4 hours, were maximum at 24 hours and had disappeared at 72 hours. These reactions consisted of erythema and induration (4-12 cm diameter at the vaccination site and 1-5 cm diameter at the skin test site) accompanied in most cases by slight local tenderness. Overall, lot BP2-WZ-2 produced somewhat smaller local reactions than BP2-PA-3, and reactions to the second dose of both vaccines were milder than those to the first dose. The local reactions were generally stronger than those usually seen with the meningococcal group A and group C polysaccharide vaccines. No correlation between reactogenicity and pre-existing antibody level to either component was observed. Volunteers were questioned after 24 hours reagrding systemic effects of the vaccines. One volunteer who received lot BP2-PA-3, indicated that he had felt slightly warm, although an oral temperature was not obtained. He, along with one of the individuals who received lot BP2-WZ-2, reported some local tenderness in the arm that received the vaccine. Throat cultures of all volunteers were initially negative for N. meningitidis and remained negative throughout the study. The stool cultures of two volunteers (6 and 7), however, were consistently positive for E. coli K1, which has a capsular polysaccharide antigenically identical to the group B polysaccharide. The results of serologic assays on pre and post-vaccination sera were very encouraging. The titers of bactericidal antibodies to the vaccine strain 99M (also called B11) rose an average of 32-fold in the volunteers that received lot BP2-WZ-2, and an average of 8-fold in those that received BP2-PA-3 (table 9). Similar rises were obtained when the sera were tested against three other group B strains with different serotype antigens. This indicated that much of the bactericidal antibody was probably directed against the group B polysaccharide. Removal

of the bactericidal activity against non-type P2 group B strains by absorption with purified E. coli K1 capsular polysaccharide, confirmed this conclusion. One volunteer in each group (3 and 8) had little or no rise in bactericidal antibodies to the B polysaccharide, but these were the two with the highest pre-existing antibody titers. The specific determinant on the group B polysaccharide against which these human bactericidal antibodies were directed, is of some interest. Since only high molecular weight B polysaccharide can inhibit these antibodies, the active determinant is clearly not the main backbone structure of the polysaccharide. This is not surprising, since the sialic acid units of the main chain have the same linkage as on the G_{D1B} and G_{T1} gangliosides. Using a sensitive complement mediated liposome lysis assay, a number of human sera were screened specifically for antibodies to the G_{T1} ganglioside. These sera included 3 convalescent sera from group B cases, several normal sera, and the two highest responders to the group B vaccine. No antibody was detected. The precise nature of the active determinant on high molecular weight group B polysaccharide is still under investigation.

In addition, to the group specific bactericidal antibodies, increases in type-specific bactericidal antibodies were also detected following appropriate absorptions to remove antibodies to the capsular polysaccharide (table 9). After absorption with a group B, type P14 strain M981, 7 of 8 volunteers had type specific bactericidal activity against the vaccine strain in their two week sera, but not in their pre-vaccination sera. Similarly, after absorption with the type P4 group C strain 118V, the sera from 6 of the 8 volunteers had type specific bactericidal antibodies against the group C, type P2 strain 138I. These antibodies were not removed by absorption with purified 99M LPS, indicating that the antibodies were most likely directed against the serotype proteins. Perhaps, most significant, is the fact that the two individuals with little or no pre-existing bactericidal antibodies, both developed significant rises in group B, specific, as well as type 2, specific bactericidal antibodies.

As a second measure of the antibody response to the protein component of the vaccine, the sera were assayed by a solid phase radioimmunoassay (SPRIA), for antibody to the vaccine strain (99M), outer membrane complex (table 10). Since the complex contains both protein and LPS, the sera were also tested for antibodies to the purified LPS as a control. Since the concentration of anti-LPS antibodies was only 3-5 percent of the

concentration of antibodies that bind to the whole complex, over 90 percent of the antibodies that bound to the complex were specific for the protein antigens. All eight volunteers had a significant increase in antibodies to the proteins. These increases ranged from 2-fold to 30-fold with a mean increase of about 10-fold. There was no significant difference in the mean response to the two vaccine lots. By means of quantitative inhibition with homologous and heterologous OMC, it was determined that a mean of 63 percent of the antiprotein antibody induced by lot BP2-WZ-2, and 47 percent of that induced by lot BP2-PA-3, was specific for the type P2 antigen.

The second dose of vaccine, given at five weeks, resulted in little or no increase in the antibody titers (tables 10 and 11). This is quite different from the response of rabbits to the vaccines. Rabbits responded weakly to the first dose, and very strongly to the second dose. Most of the antibody titers in sera, obtained at five and ten weeks, had dropped off significantly, relative to the two week and seven week sera, respectively, but remained well above the pre-vaccination level. This is likely a reflection of the fact that the bactericidal antibodies against the B polysaccharide were found to be nearly all of the IgM class. The good response obtained with the first dose and the low response produced by the second dose, suggests that only a single dose need be given in the future.

The above data clearly demonstrate the feasibility of inducing IgM bactericidal antibodies to the group B polysaccharide by vaccination, and that this bactericidal activity is effective against all group B strains, regardless of serotype. It is also significant to note that these antibodies may afford protection against infections with E. coli K1 strains, as well. Although the number of volunteers is small, it appears that lot BP2-WZ-2, gave a better antibody response to the group B polysaccharide while producing somewhat milder local reactions. The two lots produced about equal amounts of anti-protein antibody as measured in the SPRIA, but lot BP2-PA-3, appeared to induce a little more type-specific bactericidal antibody. Since the method used to prepare lot BP2-WZ-2, allows more control over the proportion and composition of the protein component of the vaccine, and results in a product with lower pyrogenicity, it has been chosen for further testing in larger numbers of volunteers.

15. It is important to understand the nature and characteristics of the antigenic determinants on the outer membrane proteins if

they are to be used as a vaccine or an important component of a vaccine against group B meningococcal disease. In a survey of normal sera, and convalescent phase sera from patients with type 2 group B, and group C disseminated meningococcal disease, bactericidal antibodies directed against the serotype proteins, were only rarely found in normal sera but were present in about two thirds of convalescent phase sera. These results indicated that the outer membrane protein antigens are capable of inducing a bactericidal antibody response in man, at least under the conditions of a natural infection. In two previous studies, type 2 outer membrane proteins that were isolated by several different procedures, were found to be poorly immunogenic in man although they had good immunogenicity in rabbit and mice. Since these two protein vaccines had been exposed to extremes of pH and/or detergents during their preparation, it seemed likely that their lack of immunogenicity was due to alteration or degradation of important antigenic determinants. This conclusion was supported by the observation that the vaccines could not inhibit the anti-protein bactericidal antibodies in human sera whereas the intact homologous outer membrane complex could.

Accordingly, studies were initiated to examine the nature, specificity and stability of the protein antigens against which human bactericidal antibodies are directed. These studies as well as vaccine studies have utilized protein serotype 2 strains since this serotype has been associated with most recent group B and C epidemics. The capacity to inhibit human antibodies specific for the serotype 2 outer membrane proteins in bactericidal assays, and in solid phase radioimmunoassays was used to follow the antigenic activity of the outer membrane proteins at various steps in their separation from the membrane.

The results fell into several categories depending on which antiserum was used in the test systems. With the first category of sera (human sera containing naturally acquired antibodies) equilibrium of type 2 outer membrane complex with 3 percent sodium deoxycholate (DOC), 0.01M EDTA, and 0.05M glycine at pH 8.8, followed by ethanol precipitation and washing, resulted in only a moderate decrease in the antigenic activity of the proteins.

Gel filtration of the DOC-dissociated complex on Sephadex G-100 in the presence of 1 percent DOC, pH 8.8 separated the complex into a protein fraction and a lipid-LPS fraction. The protein fraction alone was about 100-fold less active than the intact OMC.

Considerable activity, however, was recovered by recombining it with the lipid-LPS fraction prior to, but not after removal of the DOC by alcohol precipitation. The lipid-LPS fraction from a heterologous OMC was equally effective in reconstituting the antigenic activity of the proteins. These results suggest that naturally acquired human bactericidal antibodies specific for the outer membrane proteins are often directed against determinants which are dependent upon association of the protein with other components of the membrane (LPS) for full expression of their antigenic activity. The protein determinant against which these antibodies were directed was shown to be different than the type 2 determinant in that it was associated with a protein of lower molecular weight (≤32,000 daltons) and was not present on all type 2 strains.

With the second category of sera (human convalescent phase sera) equilibration of the OMC with 3 percent deoxycholate followed by ethanol precipitation and washing resulted in essentially complete loss of antigenic activity. Even ethanol precipitation alone resulted in a marked loss of activity. These antibodies, therefore, appeared to be directed against protein determinants which were very labile.

Sera from volunteers who received the experimental protein-polysaccharide group B vaccine lot BP2-WZ-2, and sera from rabbits immunized with viable type 2 meningococci constituted the third category. With these sera, which had clear type 2 specificity, separation of the proteins from the membrane by chromatography in the presence of sodium deoxycholate did not result in significant loss of antigenic activity. These antibodies were thus directed against a more stable determinant than the antibodies in categories 1 and 2. An earlier experimental type 2 protein vaccine which had been exposed to pH extremes, during its preparation and which lacked immunogenicity in humans failed to inhibit these category 3 antisera. The current vaccine lots (BP2-WZ-2 and BP2-PA-3), on the other hand, were fully active in the category 3 test systems.

A fourth category of sera were from rabbits immunized with vaccine lot BP2-WZ-2. The antibodies in these sera, though type 2 specific, were directed against very stable determinants which were not inactivated by exposure to both detergent and extremes of pH. Determinants reacting with these antibodies appear to lack immunogenicity in man.

In summary, human anti-OM protein bactericidal antibodies induced by natural infections with type P2 meningococci are

often directed against relatively labile determinants which do not appear to be located on the type 2 principal outer membrane protein and are inactivated to a variable extent by disruption of the normal associations of the proteins in the membrane. Although bactericidal antibodies with clear type P2 specificity were not identified in several human sera obtained following natural infections with type P2 strains, bactericidal antibodies with P2 specificity were demonstrated in human sera following vaccination with DOC-fractionated type P2 outer membrane proteins complexed to group B capsular polysaccharide. These antibodies were directed against a relatively stable determinant that was not dependent on binding to LPS for activity.

16. Studies of human immunoglobulin A (Iga) and immunity to bacterial diseases have largely been restricted to its role on secretory surfaces (secretory IgA), where its dimeric form, coupled with secretory component and the conjoining J chain, facilitate its separation and identification. Understanding of its importance within the circulation (serum IgA), where it occurs primarily as a monomer, has been hindered by the technical difficulty of completely separating serum IgA from physicochemically similar IgG. The development of immuno-adsorbent techniques to remove contaminating IgG and IgM has solved the problems of final purity, and we have recently reported that immune serum IgA, which does not activate complement, not only blocks the complement-mediated bacteriolytic activity of IgG and IgM against Neisseria meningitidis, but also may play a crucial role in the development of susceptibility to invasion by these organisms.

Extension of these data will require large-scale epidemiological studies of the prevalence and antigenic specificity of serum IgA. Purification of serum IgA for the initial studies, however, required three separate chromatographic steps which were cumbersome, time-consuming, poorly suited to small starting volumes of sera and which resulted in small yields of the purified final product. Therefore, we have developed a continuous, in-line chromatographic process involving sequential application of molecular sieve, ion-exchange immunoadsorbent-affinity and de-salting concentrating methods. The chromatographic process yields 45-55 percent (by weight) of the IgM, IgG and IgA from 1-3 ml of serum in 36 hr, each free of contamination by immunoglobulins of the other classes, and without loss or alteration of biological activity. The theoretical basis and technical details of the procedure were reported. (J. Chromatogr. 156:121, 1978).

Since mid-1975, an outbreak of disease due to group A N. meningitidis has been recognized in the Pacific Northwest. Initially, involving persons in Portland, Oregon, and Seattle, Washington, the outbreak subsequently spread north along the Pacific Coast to involve residents of Alaska. The availability of group A meningococcal vaccine, and the sensitivity of the causative organisms to sulfonamides, provided public health officials with options for control measures, but no clear guidelines for choosing among them.

Experience with group A meningococcal disease in this country has been quite limited since a major epidemic in 1945. Based on reports to the Center for Disease Control (CDC), over the previous ten years, group A had accounted for approximately one percent or less of isolates from patients with meningococcal disease. However, group A N. meningitidis continues to be a major public health problem in the "meningococcal belt" of Africa, and recently was responsible for major, widespread outbreaks in Brazil and Finland. These latter epidemics, coupled with the absence of significant disease in this country over the past 30 years, led to concern that the outbreaks in the Pacific Northwest presaged widespread disease throughout this country which would necessitate an extensive and expensive vaccination program.

On the other hand, beginning in 1970, an outbreak of group A meningococcal disease was documented in Winnipeg, Manitoba. Sixty-six patients, primarily American Indians, developed disease. The outbreak subsided without significant spread to non-Indian populations, and no group A meningococcal disease has been recognized there since late 1973. In Portland and Seattle, most of the initial cases occurred in adults who abused alcohol and/or frequented or lived in the Skid Road sections of those cities. In their restriction to a demographically definable sub-population, these outbreaks resembled previous ones in this country caused by serogroups B, C, and Y, and suggested that a far more modest vaccination and/or chemoprophylactic program, concentrated on the at-risk focus, would suffice. Consequently, a multi-center group was formed to study several related aspects of these outbreaks and to monitor the effectiveness of control measures.

Because of the similarity of this outbreak to previous focal outbreaks among military recruits at Basic Training Centers, and the expertise acquired during a decade of studying meningococcal

disease, personnel of this department were asked to coordinate this collaborative effort.

The results of the studies completed during FY 78 have been separated into four data blocks. The first includes a presentation of the cases of group A meningococcal disease in the Pacific Northwest, and a summary of clinical aspects of patients hospitalized in Seattle, and places the outbreak in Seattle within the demographic setting of Seattle's Skid Road community with particular reference to the impact of this demography on the outcome of the vaccine control program. Results of an attempt to establish a relationship between this outbreak and that in Manitoba are also included. Subsequent blocks consider (a) epidemiologic and bacteriologic studies of at-risk populations in Seattle and Portland to identify factors which might be helpful in predicting or controlling outbreaks; (b) results of lipopolysaccharide (LPS) serotyping of causative strains to determine if LPS antigens were associated with virulence; and, (c) studies of the prevalence of antibody to the group A capsular polysaccharide in at-risk and control populations, to assess the role of "herd immunity" in the epidemiology of the outbreaks. These four sets of data are summarized below.

a. Demography of the at-risk population and its effect on a vaccination control program.

In mid-1975 an outbreak of group A meningococcal disease began among Skid Road alcoholics in Seattle, Washington, spread along the Pacific Coast to Portland, Oregon, and Anchorage, Alaska, and then sporadically inland. A total of 45 cases of disease was documented. The Skid Road communities are composed of multiple, diverse populations, whose mobility, and social habits facilitate disease transmission and spread, and hinder control efforts. An important sub-population, American Indians, interchanges freely along traditional migration routes which connect with Manitoba, Canada, where the last North American outbreak of group A disease occurred among American Indians early in this decade. Vaccination campaigns were undertaken in four cities, beginning first in Seattle, where 80 percent of the target population was vaccinated. Twenty-five of 45 regional cases occurred after the start of vaccination in Seattle. Effectiveness of vaccination efforts appeared limited by difficulty in defining and reaching the at-risk population, and premature termination of vaccination efforts. Whether the lack of a regional approach or failure to use adjunctive chemoprophylaxis were contributing factors, is not known.

b. Outbreaks in Skid Road residents, and relationships between risk of disease and bacterial flow of residents.

In June 1975, and January 1976, outbreaks of group A meningococcal disease began in Skid Road communities of Seattle, Washington, and Portland, Oregon. By mid-July 1976, attack rates were 162/100,000/year in Seattle and 89/10,000/6 months in Portland. In Seattle, cases were limited to one neighborhood whose residents were younger, more mobile, and more likely to be North American Indican than other Skid Road inhabitants. A greater proportion of ill persons than Skid Road controls were heavy consumers of alcohol ($p=0.006$). Nasopharyngeal carriage of group A N. meningitidis was restricted to Skid Road habitués. Fewer Skid Road residents than non Skid Road residents carried other nasopharyngeal bacteria which interfere with the growth of group A meningococci ($p<0.05$). Strains of Bacillus pumilis and Streptococcus faecalis, which cross-react with group A, N. meningitidis were recovered from stool cultures. Colonization with cross-reacting organisms was more common among contacts of cases than Skid Road controls or non Skid Road controls (p 0.05).

c. Differentiation of the causative organism by LPS serotyping, and its relationship to other epidemic strains.

Forty strains of group A, Neisseria meningitidis, isolated from cases and carriers during a focal outbreak in the Pacific Northwest, were serotyped on the basis of antigenic differences in their lipopolysaccharides, and compared with strains from three recent worldwide epidemics. Twenty-one of 23 cases, and 13/17 carrier strains from the Pacific Northwest, were positive for serotype L10. Expression of type L10 alone, was associated with disseminated disease; expression of both L10 and L11 with nasopharyngeal carriage (p 0.00006). Type L10 strains also appeared responsible for a widespread epidemic in Brazil (6 of 6 strains), and, in part, for a focal epidemic in Manitoba (3/5), but was not found among 5 strains from an epidemic in Finland (four L11; one non-typable).

d. Increased prevalence of group-specific antibody in the at-risk population.

To assess the role of group A capsular polysaccharide antibody, in determining the extent of an outbreak of group A meningococcal disease in the Pacific Northwest, and its

relationship to the development of "risk", 313 sera from defined populations in Seattle, Washington, and 148 sera from military recruits, were assayed for antibody to the group A and C capsular polysaccharides. The population at risk, Skid Road residents and habitués, had a significantly higher prevalence of antibody to the group A polysaccharide than recruits (p 0.000001) or non Skid Road, non-alcoholic American Indians (p 0.0003). The increase in antibody prevalence was greater for group A than group C, and antedated the onset of disease. It appeared related to intimacy of contact with Skid Road environment but not ethnicity nor alcoholism, alone.

These data illustrate the complex nature of "risk" of epidemic meningococcal disease, and its importance in the development of optimal control programs. Further studies of the prevalence of bactericidal, Ig class-specific, and LPS antibody in populations in the Pacific Northwest may better define its immunological basis.

18. Previous studies of disseminated meningococcal disease conducted by this Department, have concentrated on epidemic disease in military recruits. Relatively little comparable information is available for endemic disease or for disease in civilian populations, where susceptibility is concentrated in pediatric age groups. During FY 78, two studies in civilian pediatric populations were undertaken in collaboration with Baylor University. The results are summarized below.

a) The epidemic potential of strains of N. meningitidis has been shown to be a function more of serotype than serogroup with type II strains accounting for most epidemic disease caused by groups B, C, and Y during the last decade. This had led to efforts to develop a type II protein vaccine. Endemic strains have been less extensively studied, and the current mix of serotypes causing endemic disease is unknown. Therefore, we analyzed the serotypes of three sets of endemic disease isolated: 1) 34 consecutive isolates from pediatric patients at hospitals affiliated with Baylor College of Medicine during a single 12 month period (3/77-2/78); 11 isolates from military recruits at BTC in the Southwest from 1970 to 1976, a non-epidemic period at those posts, and 3) 27 isolates from patients seen at Military Treatment Facilities (MTF) in CONUS, both military and civilian, from February, 1977 through March, 1978. Serotypes were determined utilizing the modified Gold system (2). Forty of the 72 strains were serogroup B, 14 were group C, 11 group Y,

5 group W135, and one each groups X and 29E. Among pediatric strains, 10 serotypes were represented, with an additional 7 strains being non-typable. Seven (20.6 percent) were type II. For recruits in the Southwest, 5 types were represented, with 1 non-typable strain; 4 (36.4 percent) were type II. Among contemporary MTF strains, 5 types were also represented with 3 non-typable strains; 12 (44.4 percent) of these strains were type II.

These data confirm differences in serotype distribution between epidemic, and endemic strains, and support the concept that epidemic potential is primarily a function of serotype, rather than serogroup, since epidemic strains are invariably of a single serotype, regardless of serogroup. They also raise a question as to the potential usefulness of a monovalent serotype II vaccine in preventing current endemic disease among military and civilian populations.

b. The development of the currently available meningococcal vaccines at Walter Reed was based on studied of the immune response of military recruits prior to, and following disseminated meningococcal disease, and they have proved effective in preventing disease in this age group. The vast majority of cases of meningococcal disease, both endemic, and epidemic, however, occur in infants and young children, and the capsular polysaccharide vaccines are of diminished or absent immunogenicity in infants under 2 years of age, which is the primary at-risk group. Experience in the development of these vaccines indicated to us that preparation of a potential immunogen for infants should be based upon studies of their immune response during disease, and that a minimum requirement for such a potential immunogen should be its ability to induce a reasonable, brisk, immunologic response during infection - preferably in bactericidal antibody - which is the functional correlate of immunologic protection.

Consequently, we studied acute, and convalescent sera obtained at the time of hospitalization, and 2 to 4 weeks, thereafter, from 30 consecutive patients, aged 5 weeks to 16 years, admitted to pediatric services of Baylor University in Houston, Texas, from February, 1977 through February, 1978, to determine whether, at what age, and to which antigens of the meningococcus they responded immunologically during disseminated disease.

The causative organisms were of three separate serogroups, with group B predominant, and 11 (eleven) separate serotypes,

without predominant of a single serotype. An additional seven strains were not typable using our current battery of sera. Antibody to each capsular polysaccharide was quantitated in a radioactive binding assay, and is expressed as ngm of antigen bound by 50 mcl of undiluted serum. In addition, antibody to the group B capsular polysaccharide was separately quantified by indirect hemagglutination of erythrocytes sensitized with group B capsular polysaccharide. Bactericidal antibody was measured in a radioactive bactericidal assay using a single lot of weanling rabbit serum as an exogenous source of complement. Each set of paired sera was pre-treated with penicillinase for 30 minutes at room temperature and tested, in parallel, against their homologous infecting strains. Results are expressed as the titre of each serum capable of killing 50 percent of 10^7 organisms.

Antibody response to the group C capsular polysaccharide appeared age related, with children over 2 years of age responding briskly with a geometric mean increase of 100 ngm in antigen binding capacity; while one infant, aged 4 months responded only marginally, at best. These findings are consistent with the non-immunogenicity of the group C polysaccharide vaccine in children under 2 years of age. A single 10 year old child responded appropriately to the group Y capsular polysaccharide.

In contrast, responses to the group B capsular polysaccharide were inconstant and meagre, indeed. Because of the low binding capacity of these sera, near the limit of sensitivity of the assay, individuals who evidenced at least a 1 ngm rise in binding capacity, plus a 4-fold increase in hemagglutination; or a greater than 2 ngm rise in binding capacity alone were considered to have responded. Even using this generous criterion, only 7 of 24 (29 percent) individuals were judged to have responded. Response appeared unrelated to age (=.48 by x^2 analysis with 1 d.f.), and even among responders was quite muted, with a geometric mean increase in binding capacity of only 2.7 ngm, and a range of 1.4 to 4.9 ngm bound. Using the same criterion, 7 of 9 (78 percent of military recruits, infected with group B strains, and tested at the same time, responded with a geometric mean increase of 14.7 ngm, and a range of 28.65 ngm antigen bound. Surprisingly, all children developed bactericidal antibody, regardless of infecting group or type. Bactericidal antibody response was greater for group B cases than for group C (geometric $\log_2$, mean titre 1:77 vs 1:46), and among group B cases, greater in children over 2 years of age (mean titre 1:157) than in those under 2 years of age (mean titre 1:34). Although the numbers are small, bactericidal antibody response

appeared greater in patients who did not, but was unrelated to the serotype of the infecting strain.

Outer membrane complex lipopolysaccharides or LPS, extracted from the group C, type II, III, and IV prototype strains by the hot phenol-water technique were used in attempts to inhibit the bactericidal activity of selected sera, in order to determine the antigenic specificity of bactericidal antibody in children who did not respond to the capsular polysaccharide. The three group C strains, in addition to differences in serotype, elaborate antigenically distinct lipopolysaccharides. Their extracted LPS's were free of capsular polysaccharides, protein or nucleic acids, and were anti-complementary only in quantities in excess of 20 µgm total inhibitor. Convalescent sera from 5 children, 4 infected with Group B types V, IX and NT strains, and 1 with Group C, type VIII strains, were diluted to the break-point in the titre-curve for lysis of their homologous strains, and the inhibitory capacity of each LPS determined in parallel, by addition of from 625 ngm to 10 mcgm of total inhibitor to the reaction mixture 1 hour prior to the addition of complement, and meningococci, followed by shaking at ambient temperature.

Each of the 5 sera was inhibited by one or more lipopolysaccharide. Two distinct patterns were observed: One in which the three LPS preparations were of equal inhibitory capacity, and one in which the Group C, type III strains's LPS was either the only inhibitory preparation or was 4 to 5-fold more inhibitory - on a weight basis.

Immune response following disseminated meningococcal disease appears to be predictive of vaccine efficacy. Since infants less than 6 months of age respond adequately with bactericidal antibody directed at LPS antigens, but only fitfully to meningococcal capsular polysaccharides, vaccination of the high-risk group is theoretically feasible with the appropriate non-capsular antigen.

19. Strains of meningococci of serogroups X, Y, Z, 29E and W135, have historically accounted for little, if any, epidemic disease. They do, however, continue to contribute significantly to overall morbidity, with from 15-50 percent of cases, both civilian and military, being of these serogroups during endemic periods. Furthermore, relatively small outbreaks of Group Y meningococcal disease are frequent among military recruits, and Group W135 has emerged as a significant pathogen in Northern

Europe. If the goal of total prevention of meningococcal disease among military recruits is to be reached, vaccines capable of preventing dissemination of these "minor" serogroups from the nasopharynx must be developed.

During FY 78, capsular polysaccharide vaccines against Groups Y, W135 and 29E were prepared and tested in laboratory and recruit volunteers at the WRAIR and Fort Dix, New Jersey. Analysis of immunogenicity data is incomplete, however, all three vaccines were immunogenic in laboratory volunteers, and were well tolerated by both groups of volunteers.

Two additional studies were also undertaken during FY 78, to provide background date for the use of these vaccines.

a) Group Z, Neisseria meningitidis: Immunochemical analysis of the group-specific capsular polysaccharide and its "one-way" cross reaction with Group 29E capsular polysaccharide.

The chemical composition of the group-specific capsular polysaccharide of Group Z, Neisseria meningitidis was determined by gas-liquid chromatographic analysis, and the nature of its immunological cross-reaction with the Group 29E capsular polysaccharide investigated using bacterial agglutination, precipitin-in-gel, primary antigen binding, and immune lysis assays. Group Z, capsular polysaccharide consisted of a homo-polymer of N-acetyl galactosamine (Gal-NH_3), while 29E capsular polysaccharide consisted of a co-polymer of Gal-NH_3, and 2-keto, 3-deox octulosonic acid (KDO). An immunological cross reaction was observed between the two polysaccharides, presumably involving the Gal-NH_3 portions. Demonstration of the cross-reaction between Group Z antisera, and the Group 29E polysaccharide varied with the assay used. It was most readily apparent in assays of immune lysis, least apparent when precipitins were sought in gel, and inapparent when bacterial agglutination, the standard assay for determining serogroup, was employed. The cross-reacting determinant appeared to be 10-fold more prevalent with the Group 29E polysaccharide than in the Group Z polysaccharide.

b) Disease due to serogroup W134, Neisseria meningitidis.

Since its description in 1968, serogroup W135, Neisseria meningitidis has been considered a "minor" meningococcal serogroup

of little clinical importance (originally denominated "135", the "W" has subsequently been appended to indicate its origin at Walter Reed). Beginning in 1975, however, this serogroup emerged as a significant cause of disease in Northern Europe, accounting for 10-13 percent of all cases of disseminated meningococcal disease in Belgium and Scotland. Although still little recognized in this hemisphere, the European experience has raised fears that it may also emerge as a significant serogroup here.

Recently, three cases of disseminated meningococcal disease due to this serogroup were reported from a single city. Although two of the cases had documented meningococcal bacteremia, and the final case had meningococcal pyoarthrosis and, therefore, can be assumed to have had antecedent bacteremia, disease was "mild", in all cases, and meningitis did not supervene. The relatively benign nature of these illnesses, and the dearth of other clinical experience with this organism lead us to wonder whether disease due to this serogroup might not be different from that caused by the more common "major" serogroups.

From December, 1977, through May, 1978, at least five patients, ranging in age from 16 months to 23 years, seen at U.S. Army-Medical Treatment Facilities in this country, have had documented disseminated disease due to serogroup W135, N. meningitidis. The vagaries of reportage from so far-flung a patient catchment area do not permit epidemiological conclusions; however, these five cases in six months represent as many as have been seen in the prior ten years, combined, among army personnel, and their dependents.

These cases clearly demonstrate that serogroup W135, N. meningitidis is fully pathogenic for man. The clinical spectrum of disease included uncomplicated, and easily treated meningitis without rash or evidence of meningococcal septicemia; mild meningococcemia with petechial rash, and meningitis; severe meningitis with residual cranial nerve palsy; fulminant meningococcemia with disseminated intravascular coagulation, shock, and acute renal failure leading to infarction of the extremities, and loss of the terminal digits; and meningococcal pneumonia during an outbreak of acute viral respiratory disease, complicated by fulminant meningococcemia with disseminated intravascular coagulation, and death. In addition, one case may have manifested post-treatment serum sickness on day 11, due to antibody formation to the capsular polysaccharide, a complication reported in about 5 percent of patients with disease due to other serogroups. When combined with previously reported cases,

the full spectrum of disease caused by other serogroups of N. meningitidis, from a mild and non-specific, febrile illness which may be self-limited to fulminant, and rapidly fatal sepsis with circulatory collapse, and encephalitis, is represented.

Although epidemiological significance cannot be attached to these cases, their clustering may not be fortuitous, in view of recent European experience. Nor should significance be attached to the fact that milder disease occurred in younger patients. The full spectrum of disseminated meningococcal disease, from mild, self-limited, respiratory infection to catastrophic collapse and death, is routinely seen in the military recruit population.

The use of rifampin to prophylactically treat "contacts" of three of these patients was the decision of the primary physician. Four of the five strains were sensitive to sulfadiazine, and this antibacterial agent would have been a suitable alternative for prophylaxis of intimate contacts of the patients they infected. However, one strain was resistant to sulfadiazine, which emphasizes that, as with all strains of N. meningitidis, sulfadiazine sensitivity cannot be invariably inferred from their serogroup.

20. IgA, purified from sera of patients convalescing from group C, meningococcal disease, although not bactericidal in cooperation with complement, can induce monocyte-mediated antibacterial activity against Group C, meningococci (Fig. 1). This cell-mediated function of IgA is comparable to that of similarly purified IgG in terms of its a) antigenic specificity for the homologous capsular polysaccharide (Fig. 2), b) 15-30 minute test incubation lag time of reactivity (Fig. 3), and c) 37°C optimal temperature requirement (Fig. 4). IgA appears to be more sensitive than IgG in our system since effective amounts of IgA bind 4-fold less specific antigen than that required by IgG (Fig. 5). These data suggest that IgA may participate in antibacterial monocyte-mediated host immune defenses particularly where complement levels are low or ineffective.

21. Culture methodology was developed in order to measure the kinetics, and cellular interactions which govern the ability of human peripheral blood lymphocytes (PBL) to produce IgG, IgM or IgA directed against specific protein, and polysaccharide bacterial antigens. Following in vivo immunization with tetanus toxoid (TT), and group C, meningococcal polysaccharide (Csss), cells capable of producing specific Ig (in the absence of in vitro stimulation), were detectable among PBL only on days 5-9 post-immunization. When pokeweed mitogen (PWM) was used to

mature B-lymphocytes into Ig-secreting plasma cells in vitro, cells capable of producing specific Ig were detectable throughout the three-week post-immunization study period. The days in which peak Ig production occurred tended to vary in cycles which were usually asynchronous depending upon which Ig class, and which specific antigen was under investigation. (Figures 6 and 7). Three lines of evidence indicate that the Ig detected was synthesized, and secreted by the cultured cells, and not merely carried by the cells from donor serum:

a) Known methods of inhibition of Ig synthesis (irradiation, actinomycin-D, puromycin and mitomycin-C), inhibited Ig production 85 percent to 100 percent. PHA was also an effective inhibitor of many types of Ig production. (Figures 8 and 9).

b) The amount of Ig secreted increased as the in vitro culture period was lengthened.

c) In the absence of PWM, Ig production was undetectable even when specific Ig levels in donor sera were high (12-19 days, post-immunization).

Our data indicate that following in vivo immunization, human peripheral blood lymphocytes can be used in vitro to study the kinetics, and cellular interactions which govern the production of specific IgG, IgM and IgA directed against defined protein, and polysaccharide bacterial antigens. Use of similar assays has previously advanced the cellular foundation of murine humoral immunology. Application of our new methodology using readily available human peripheral blood cells will allow sophisticated analysis of the cellular basis of modulation, and immunopotentiation of specific antibacterial antibody responses in humans.

Pneumococcal antiserum is opsonic in vitro, and using a bactericidal assay employing human polymorphonuclear leukocytes, complement, and rabbit antisera we have found that antisera raised against Streptococcus pneumonia, type 14 (SP-14), are opsonic Group B, type III, streptococci (GBS III)(Figure 10 and Table 11). The antisera were also protective in an experimental rat model of neonatal GBS-III sepsis (Fig. 11). An-S. pneumoniam type 3 (SP-3), antisera were ineffective both in vitro (Table 12), and in vivo (Fig. 11). Opsonic activity against GBS-III was abrogated by absorption of either anti-GBS III antisera or anti-SP-14 antisera with either GBS-III or SP-14 organisms, but not when SP-3 or group B strain 090R streptococci were used in

adsorptions. (Table 13). Since 090R streptococci express group-specific but not type-specific antigens, our adsorptions data indicate that the antibody in anti-SP-14 antisera, which opsonizes GBS-III is directed against the type-specific capsular polysaccharide of GBS-III. Since the commercially available pneumococcal vaccine has been shown to induce anti-SP-14 anti-SP-14 antibodies in humans, and since invasive neonatal GBS disease has been associated with a deficiency of maternal type-specific antibody, neonatal protection against GBS-III disease (which has recently emerged as a dominant cause of morbidity and mortality) may be possible by maternal immunization with this vaccine.

Table 1 Bactericidal and pyocin typing of strains isolated from consorts

Strain	Bactericidal typing: Antisera[1] A B	C D E	F G H I	J K	L M N	O P	Q R
Consort 1 084 12 Feb	- -	+ ± +	+ + - +	+ +	- - +	+ +	+ +
127 25 Mar	- -	+ + +	+ + - +	+ +	- - +	+ +	+ +
Consort 2 093 13 Feb	- -	+ + +	+ + - +	+ +	- - +	+ +	+ +
130 26 Mar	- -	+ + +	+ + - +	+ +	- - +	+ +	+ +

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Table 1 Continued

Strain	Pyocin typing: Inhibition pattern(2) B	C	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	V
Consort 1 094 12 Feb	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
127 25 Mar	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Consort 2 093 13 Feb	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0
130 26 Mar	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(1) + = killing 1:4, ± = killing 1:1 titer, (-) = no killing. The same killing pattern in 19 out of 20 s must be the same to be considered identical strains.
(2) + = killing by pyocin; ± = light growth; 0 = no killing; n = not tested

Table 2 Inhibition of Epithelial Cell Attachment (IEA) by Rabbit Antisera

Antisera[2]	IEA titer[1] Strain 093	130	084	127
084 (12 Feb)	1:32	1:4	1:128	1:4
127 (25 Mar)	1:2	1:256	1:4	1:256
093 (13)	1:128	1:8	1:128	1:2
130 (26 Mar)	1:16	1:64	1:16	1:64
093 abs 130[3]	1:16	1:1	1:32	1:2
130 abs 093[3]	< 1:1	1:64	< 1:1	1:8

(1) 50% inhibition
(2) antisera raised to piliated colonies
(3) antisera 130 and 093 absorbed with strains 093 and 130, respectively

Table 3 Cross reactivity of pili as determined by solid phase radioimmunoassay

	pili			
	084		127	
antisera	ab. bound(1)	%(2)	ab. bound	%
084	552 µg/ml	100%	130 µg/ml	24%
127	69 "	52%	132 "	100%
093	534 "	100%	123 "	23%
130	58 "	33%	176 "	100%
093 abs 130	520 "	100%	40 µg/ml	8%
130 abs 093	15 µg/ml	16%	97 µg/ml	100%
084 abs 127	587 "	100%	40	8%
127 abs 084	ND		ND	

(1) antibody bound (µg/ml) to pili as estimated by quantitative precipitation with meningococcal outer membrane complex.

(2) percent binding of antibody to pilus antigen. The amount of antibody bound to pili isolated from the homologous strain was considered 100% binding.

Table 4 Binding of antibody in vaginal secretions to gonococcal lipopolysaccharide (LPS), pili, and native cell wall complex (NC)

	PERCENT ANTIBODY BOUND			IEA
Vaginal secretion	LPS	Pili	NC	
unabsorbed	24.9	12.0	26.0	1.32
abs LPS	0	13.6	9.7	1.32
abs alum	6.0	14.3	13.9	1.32
abs pili	4.6	0	2.0	<1:1
abs NC	1.5	0	0	< 1:1
abs LPS, then pili	0	0	ND	< 1:1
abs LPS, then NC	0	0	0	<1:1
normal secretion	1.7	2.4	0	< 1:1

Table 5 Inhibition of Epithelial Cell Attachment by Vaginal Secretion Absorbed with Homologous Lipopolysaccharide, Native Complex or Pili

Dilution	Unabsorbed	LPS	NC	Pili
1:1	95%	92%	48%	17%
1:2	92	90	38	20
1:4	88	86	42	20
1:8	81	81	38	9
1:16	75	76	38	20
1:32	55	68	36	14
1:64	42	59	32	13
1:128	32	36	32	14
1:256	21	34	28	9
1:512	13	29	28	13

Table 6 Sensitivity of bacterial strains to various thiosemcarbazone compounds

MIC's/µg/ml

Thiosemi-carbazone	Staph	Gp D	Shig	E coli	Pseud	Prot	Klb	N gon	N men
A(1)[a]	2-8	>8	>256	>256	>256	>256	>256	.031-.062	1
B(2)		>8	128	128	128-256	128	128-256	.031	.062
C	>8	>8	>256	>256	>256	>256	>256	.031-0.125	>1
D(1)	0-5	2-8	>256	>256	>256	>256	>256	.031-0.62	0.25
E	>8	>8	512	>512	>512	>512	>512	2-16	>1
F	8	8	>512	>512	>512	>512	>512	0.031	>1
G	4-8	4	>512	>512	128-512	>512	>512	.062	>1
H(4)	2-8	4	64-128	128	128	128	256	< .5	.31
I(I)	>16	>16	>512	>512	>512	256-512	256-512	< 15	.125
J	8-16	16	>512	>512	>512	>512	>512	0.031	.25
K	>16	>16	>512	>512	>512	>512	>512	.031	.062
L(8)	1	2	64	6	12	2-128	6-128	.031	< .031

Table 6 Sensitivity of bacterial strains to various thiosemicarbazone compounds (Cont'd)

M(2)	>16	16->16	64-512	512	>512	512	256-512	<.15	.25
N	2	16	256-512	>512	>512	>512	>512	.125	.125
O(7)	.5	1-4	64-212	6	6-256	6	4	0.062	.062
P	>16	>16	128-256	512	256	256	256-512	.062	>1
Q	>16	>16	128-512	>512	>512	128-256	212-512	.125	>2
R(5)	>16	>16	64-128	6	256-512	64-128	64-256	.15	.062
S(2)	2	1-2	>512	>512	>512	256-512	256-512	.062	.25
T	8-16	>16	128-256	128	>512	128-512	128-256	.062	.5
U	>16	>16	>512	>512	>512	>512	>512	.062	.5
V(2)	4	>16	>512	>512	6-128	>512	>512	.15	.5
W	4-8	>16	128	256	125-256	256-512	128-256	.031	72

[a] ( ) = no of tests performed

Table 7 Immunochemical relationship between diptheria toxin and P. aeruginosa Toxin A.

Reaction	Inhibitory Antigen	Percent Inhibition
Antitoxin A vs Toxin A	Toxin A (PA)	92.3%
Antitoxin A vs Toxin A	Diptheria Toxin (DT)	14.9%
Antitoxin A vs Toxin A	Trypsin treated DT	93.6%
Antitoxin A vs Toxin A	Fragment A of DT	80.0%
Anti DT vs DT	DT	92.3%
Anti DT vs DT	Trypsin treated DT	95.6%
Anti DT vs DT	Toxin A	5.4%

Table 8 Sensitivity[a] of R-mutants and parent strains to pyocins

	Pyocins																			
R-Type	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	R	S	T	W
SF-1167 Re	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF-1117 Rd_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF-1121 Rd_1P^-	0	0	0	+	0	+	0	+	+	+	0	0	0	0	0	0	0	0	0	0
SF-1286 Rd_1P^+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF-1119 RcP^-	+	+	+	0	+	0	+	0	0	0	+	0	+	+	+	+	+	+	+	0
SF-1195 RcP^+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF-1127 Rb	+	+	+	0	+	0	+	0	0	0	+	±	+	+	+	+	+	+	+	0
SF-1112 Ra	+	+	+	+	+	+	+	±	0	+	+	0	+	+	+	+	+	+	+	0
SF-1111 parent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] +, killing; ±, light overgrowth; 0, no killing

Table 9 Bactericidal antibody response of individual volunteers to the first dose of vaccine. Reciprocal bactericidal titer vs indicated test strain

Vaccine lot	Vol. number	99M		Group B* non type P2		Group C type P2†		99M‡	
		Pre	Post**	Pre	Post	Pre	Post	Pre	Post
BP2-WZ-2	1	0	512	1.6	315	0	64	0	64
	2	64	2048	8	1024	0	0	0	64
	3	256	256	52	128	0	0	0	0
	4	64	4096	10	1260	0	8	0	16
BP2-PA-3	5	8	128	0	26	0	128	0	128
	6	32	256	16	256	0	2	0	2
	7	64	256	13	128	0	512	0	256
	8	128	256	32	79	0	4	0	2

* Geometric mean of titers against strains 6155(B:P9:L3,7), M136 (B:P4,7:L4), and M1080(B:P11:L3,4,7,8).

† Sera absorbed with 118V(C:P3,4:L2,4) were tested against 138I (C:P2:L3)

‡ Sera absorbed with M981(B:P14:L5)

** Two weeks post vaccination

Table 10 Antibody response to the protein component of the vaccine as determined by SPRIA.

Vaccine Lot	No. of Volunteers	Antigen*	Serum (week)	Mean Antibody Conc.†	Range
BP2-WZ-2	4	OMC	0	5.7	1.7-14
			2	54	17-165
			5	40	18-57
			7	40	18-77
			10	35	15-60
		LPS	0	0.71	0.52-0.92
			2	0.96	0.52-3.4
			5	0.78	0.50-2.2
			7	0.8	0.47-1.8
			10	0.76	0.37-1.9
BP2-PA-3	4	OMC	0	4.5	1.5-11
			2	46	23-93
			5	33	16-75
			7	35	16-79
			10	31	12-66
		LPS	0	1.0	0.5-3.5
			2	1.9	1.3-3.0
			5	1.8	1.2-3.5
			7	1.5	0.5-3.6
			10	1.4	0.5-3.1

* Antigens were derived from the vaccine strain.
† µg antibody/ml.

Table 11 Duration of human bactericidal antibody response and effect of booster dose*.

Vaccine Lot	Number of Volunteers	Serum (week)	Test strain 6155 (B:P9:L3,7)	Test strain 138I† (C:P2:L3)
BP2-WZ-2	4	0	14‡	0
		2	512	2.8
		5	181	3.5
		7	274	4.0
		10	128	3.5
BP2-PA-3	4	0	5.7	0
		2	79	28
		5	56	20
		7	79	16
		10	23	8

* Second dose was given at five weeks.
† Sera were absorbed with 118V(C:P3,4:L2,4).
‡ Geometric mean reciprocal titers.

Table 12 The effect of low-titer rabbit antisera on PMN bactericidal activity against GBS III.

Serum	Serum dilution	No. of viable bacteria/ml x 10^6			PMN bactericidal activity*	
		0 min	60 min	120 min	60 min	120 min
None	0	8.6	17.2	30.4	0%	0%
Pre-	1/2.5	6.2	17.2	27.6	0%	0%
immune	1/5	7.0	14.0	22.0	0%	0%
	1/10	6.4	16.8	28.0	0%	0%
	1/20	6.0	16.4	22.8	0%	0%
	1/40	6.5	13.2	24.4	0%	0%
Anti-	1/2.5	7.3	0.3	0.1	96%	99%
GBS III	1/5	9.6	1.7	0.2	82%	98%
	1/10**	19.2	1.2	0.8	94%	96%
	1/20†	14.0	3.7	6.0	74%	57%
	1/40	24.0	16.4	9.2	32%	61%
	1/80	11.2	6.5	30.8	0%	0%
Anti-	1/2.5	9.8	1.2	1.5	99%	85%
P 14	1/5**	14.8	1.9	0.8	87%	95%
	1/10	11.7	6.2	6.9	47%	41%
	1/20†	15.6	7.1	4.9	54%	69%
	1/40	8.0	6.7	8.6	16%	0%
	1/80	6.8	8.9	10.4	0%	0%
Anti-	1/2.5	7.0	3.2	64.0	55%	0%

Table 12 Continued

P 3	1/5	18.8	16.0	77.2	11%	0%
	1/10	14.4	9.2	85.6	36%	0%
	1/20	13.2	78.8	80.0	0%	0%
	1/40	11.6	30.8	80.0	0%	0%

Abbreviation as follows: GBS III-Group B Streptococci Type III, P 14-S, pneumoniae Type 14, P 3-S, pneumoniae Type 3, PMN-Polymorphonuclear neutrophiles

* Per cent bacteria killed compared to no. of viable bacteria present in the initial inoculum (0 min) according to the formula shown in Materials and Methods.

** Highest titer at which > 80% of bacteria killed following both 60 and 120 min. of test incubation.

† Highest titer at which >50% of bacteria killed following both 60 and 120 min. of test incubation.

Table 13 The effect of adsorption with homologous or heterologous bacteria on the ability of rabbit antisera to opsonize GBS III.

Anti-serum	Adsorbing bacteria	No. of viable bacteria/ml x 10^6		PMN bactericidal activity against GBS III
		0 min.	120 min.	at 120 min.*
P 14†	None	4.3	0.2	95%
	P 14	6.6	>8.0	0%
	GBS III (Nor)	10.4	28.0	0%
	GBS III (620)	5.0	>8.0	0%
	GBS 090R	5.6	0.7	87%
	P 3	18.0	0.4	98%
GBS III** (Nor)	None	7.5	0.04	99%
	GBS III (Nor)	5.8	>8.0	0%
	P 14	31.2	54.8	0%

Abbreviations as follows: GBS III-Group B Streptococci Type III, GBS 090R-Group B Streptococcus Strain 090R, P 14-S, pneumoniae Type 14, P 3-S. pneumoniae Type 3, PMN-Polymorphonuclear neutrophiles

* Per cent bacteria killed compared to no. of viable bacteria in the initial inoculum (0 min.) according to the formula shown in Materials and Methods.

** Serum titers used = 1/10

† Serum titers used = 1/20

Figure 1

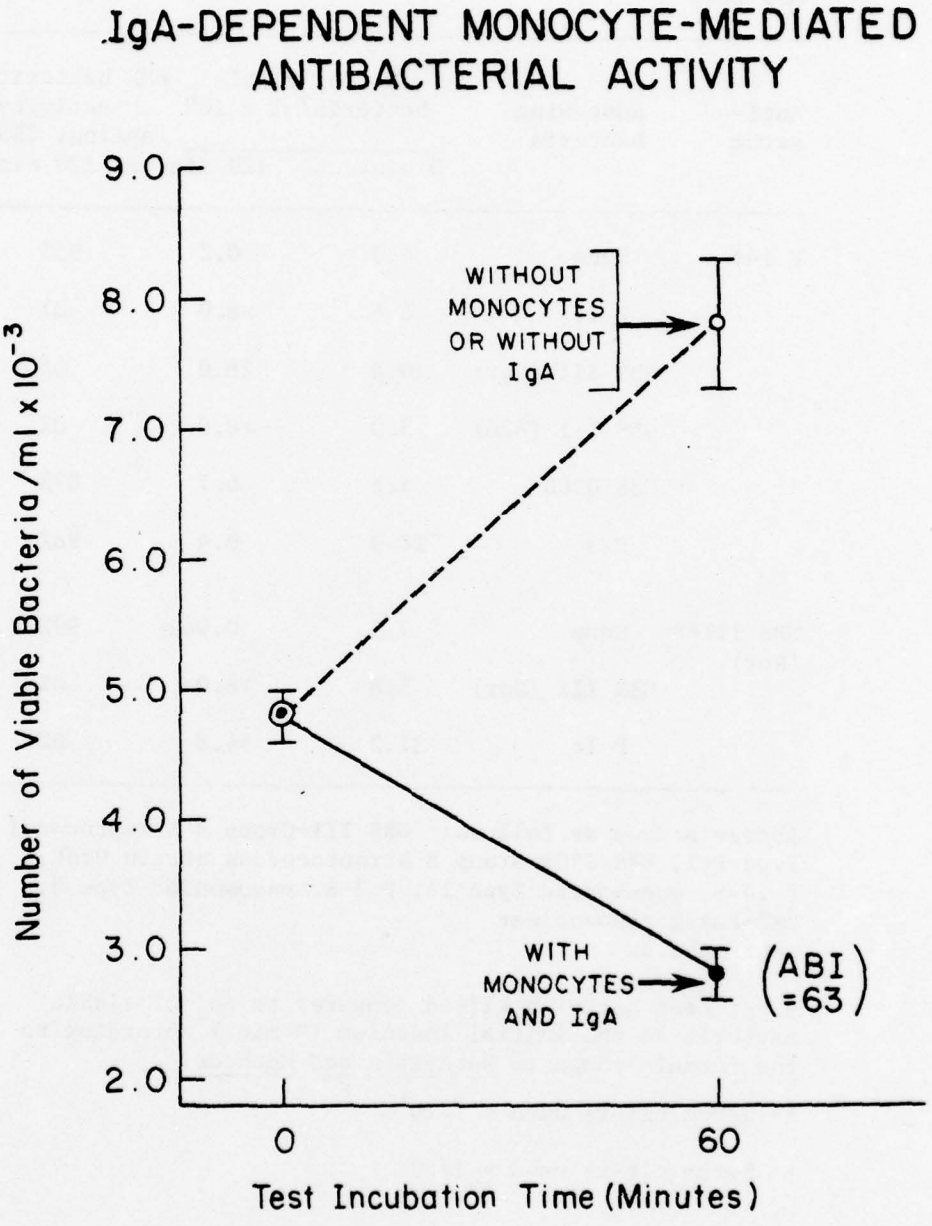

IgA-DEPENDENT MONOCYTE-MEDIATED ANTIBACTERIAL ACTIVITY
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
Number of Viable Bacteria/ml x 10^{-3}
WITHOUT MONOCYTES OR WITHOUT IgA
WITH MONOCYTES AND IgA
(ABI =63)
0
60
Test Incubation Time (Minutes)

Figure 2

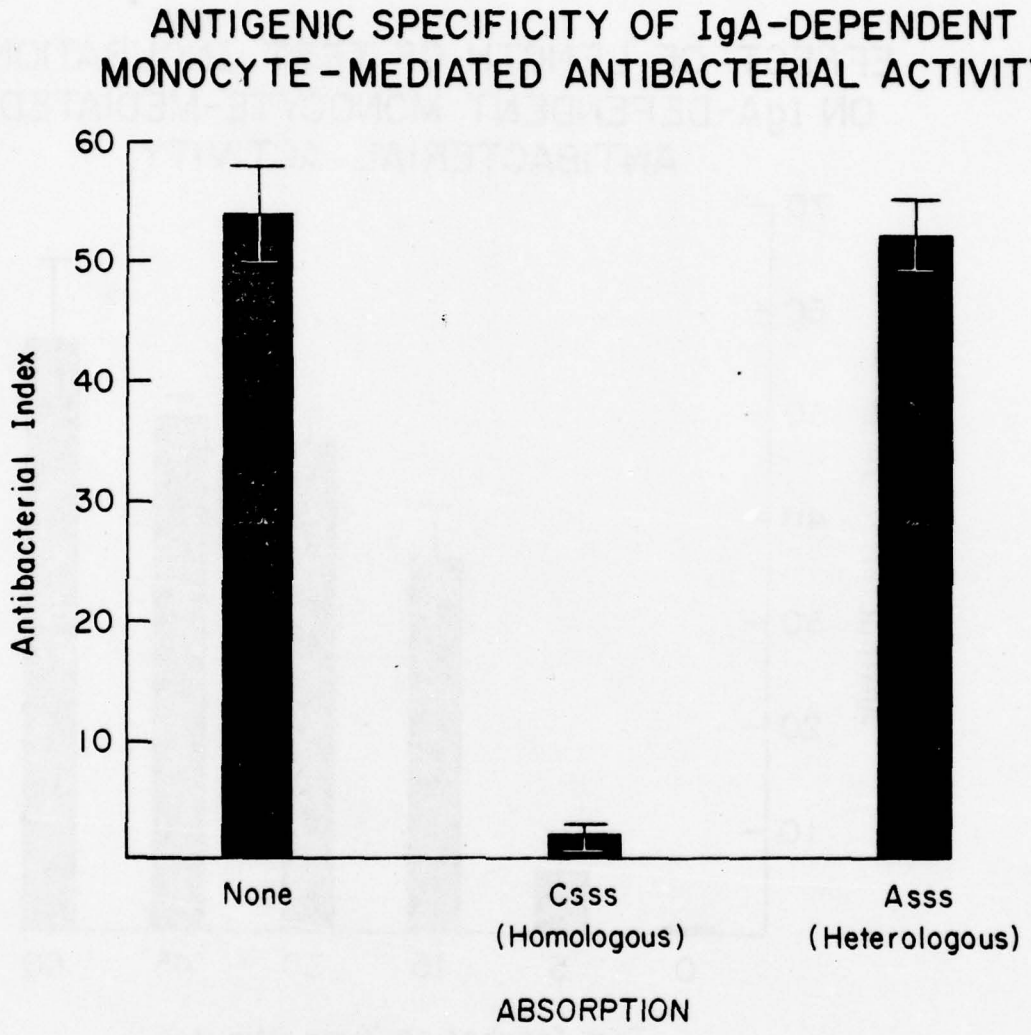

ANTIGENIC SPECIFICITY OF IgA-DEPENDENT
MONOCYTE-MEDIATED ANTIBACTERIAL ACTIVITY
Antibacterial Index
60
50
40
30
20
10
None
Csss
(Homologous)
Asss
(Heterologous)
ABSORPTION

Figure 3

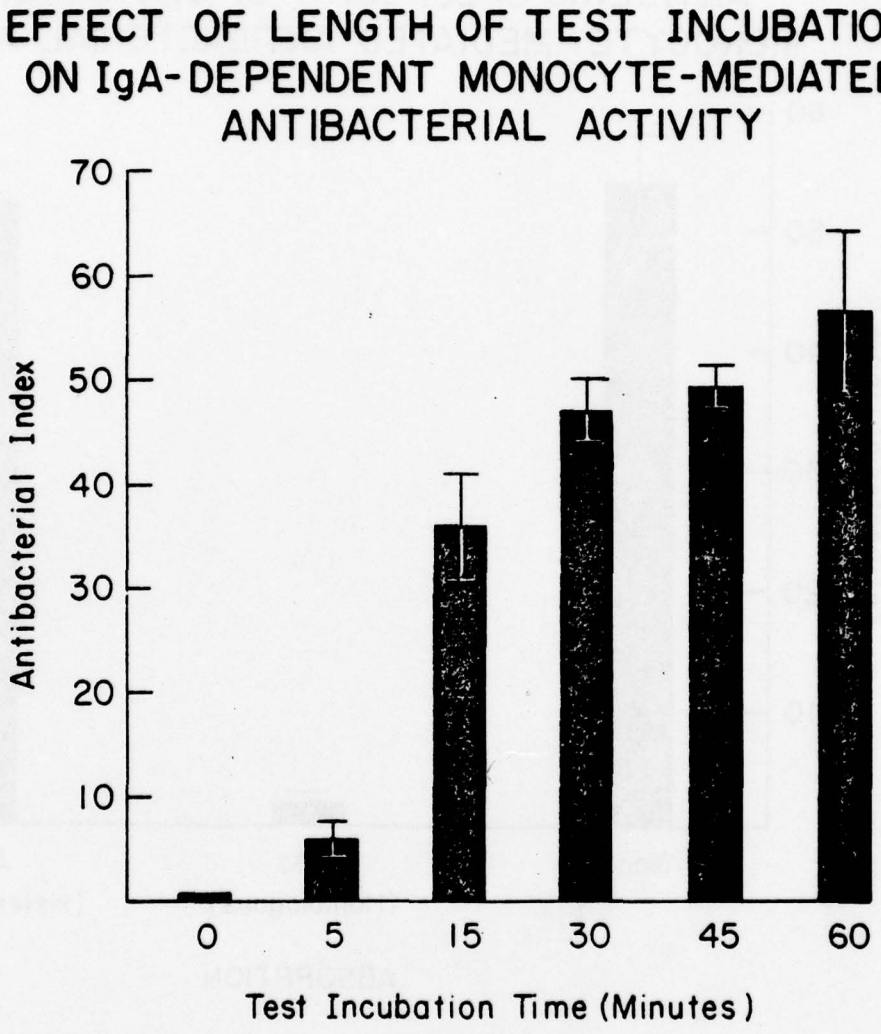

EFFECT OF LENGTH OF TEST INCUBATION ON IgA-DEPENDENT MONOCYTE-MEDIATED ANTIBACTERIAL ACTIVITY
Antibacterial Index
70
60
50
40
30
20
10
0
5
15
30
45
60
Test Incubation Time (Minutes)

Figure 4

EFFECT OF TEST INCUBATION TEMPERATURE ON IgA-DEPENDENT MONOCYTE-MEDIATED ANTIBACTERIAL ACTIVITY

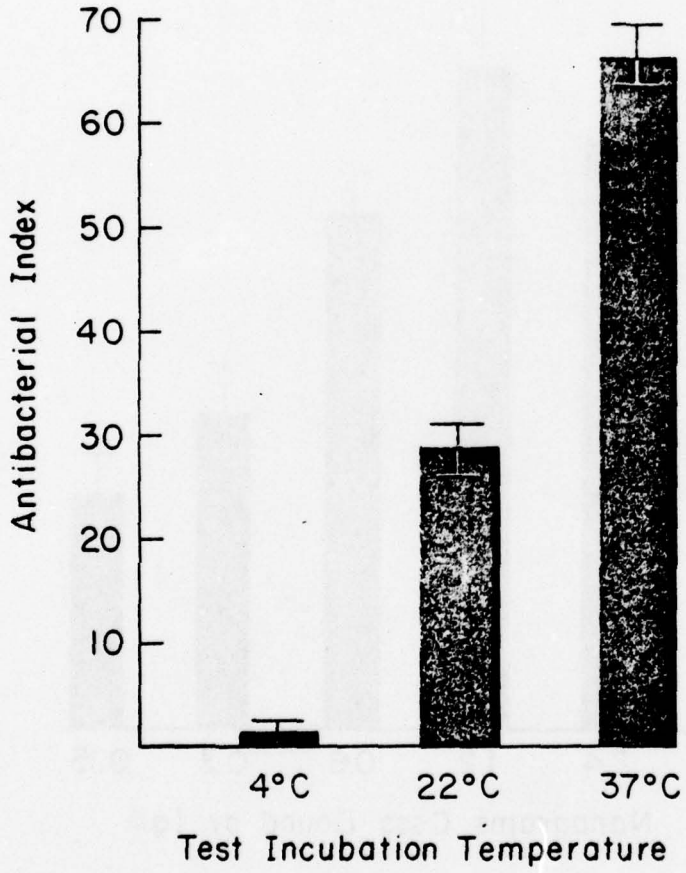


Figure 5

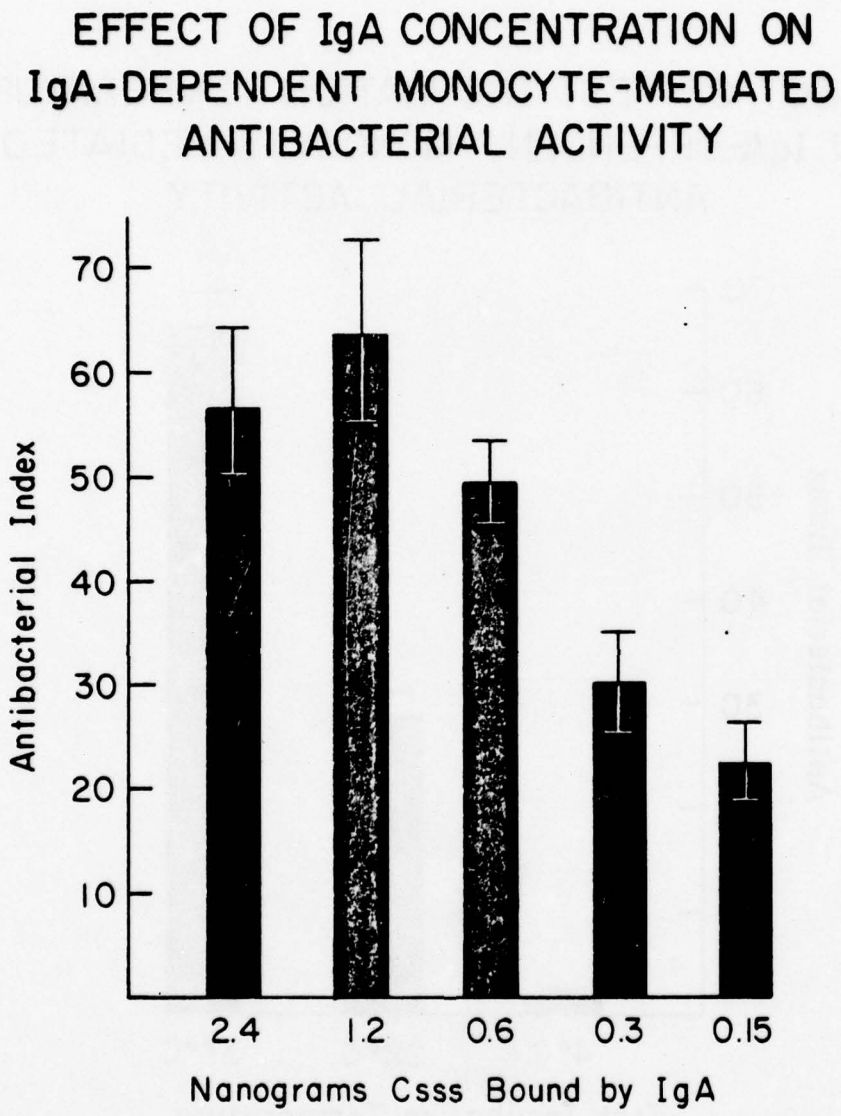

EFFECT OF IgA CONCENTRATION ON
IgA-DEPENDENT MONOCYTE-MEDIATED
ANTIBACTERIAL ACTIVITY
Antibacterial Index
70
60
50
40
30
20
10
2.4
1.2
0.6
0.3
0.15
Nanograms Csss Bound by IgA

Figure 6

IN VITRO SECRETIONS OF ANTI-Mgc IgG, IgM & IgA BY HUMAN PBL FOLLOWING IN VIVO Mgc Csss IMMUNIZATION

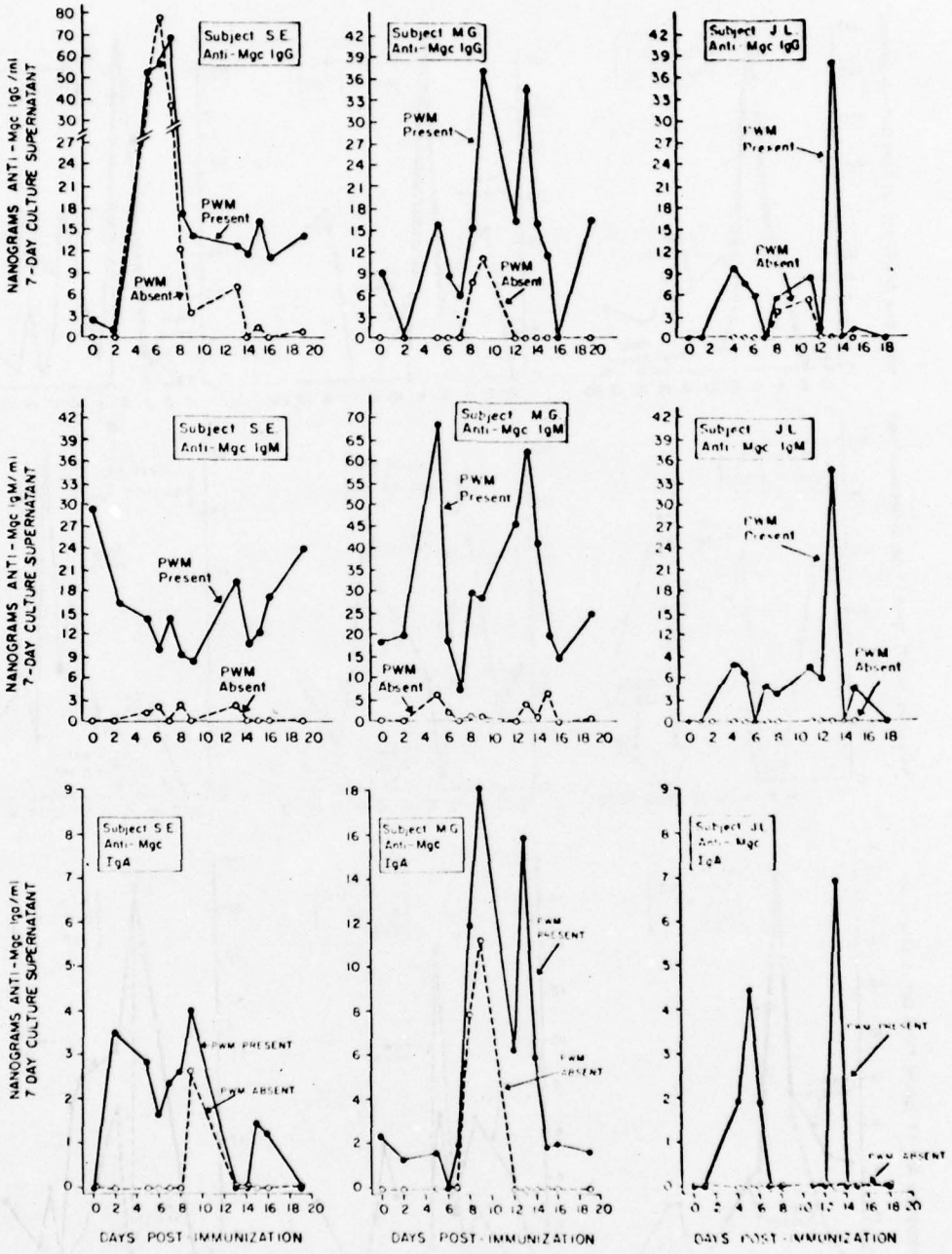


Figure 7

IN VITRO SECRETIONS OF ANTI-TT IgG, IgM & IgA BY HUMAN PBL FOLLOWING IN VIVO TT IMMUNIZATION

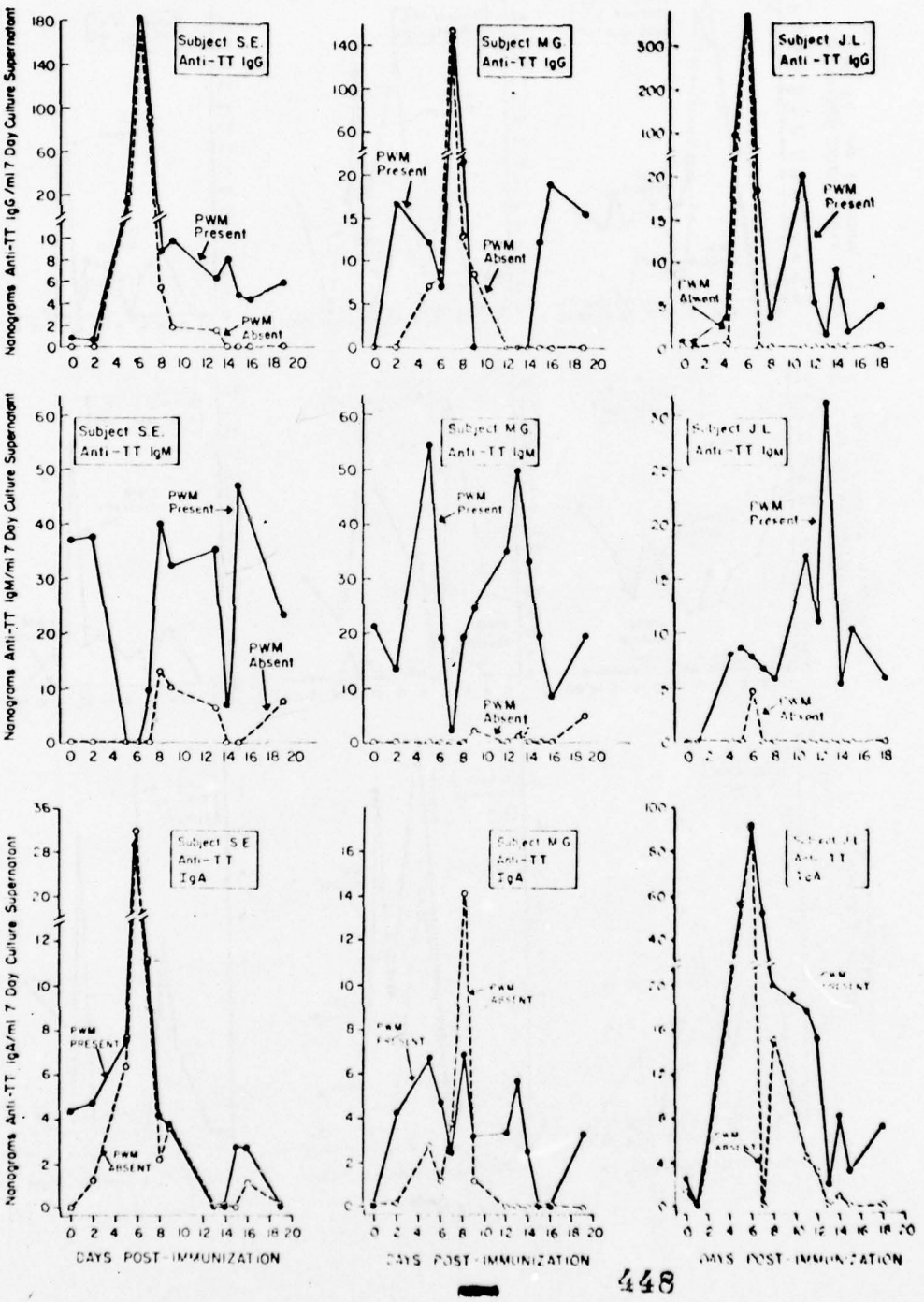
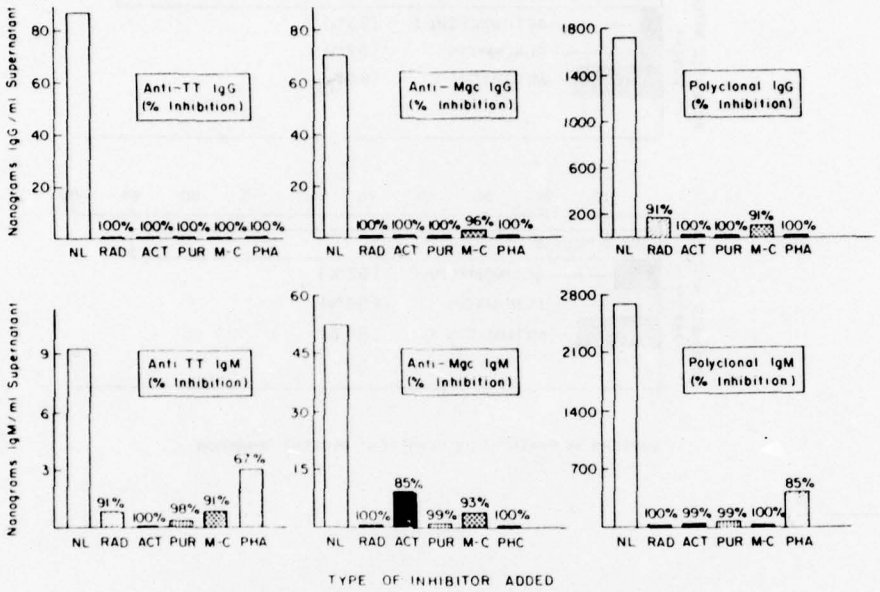


Figure 8

THE EFFECT OF INHIBITORS OF ANTIBODY SYNTHESIS ON Ig SECRETION BY HUMAN PBL CULTURED FOR 7 DAYS WITH PWM



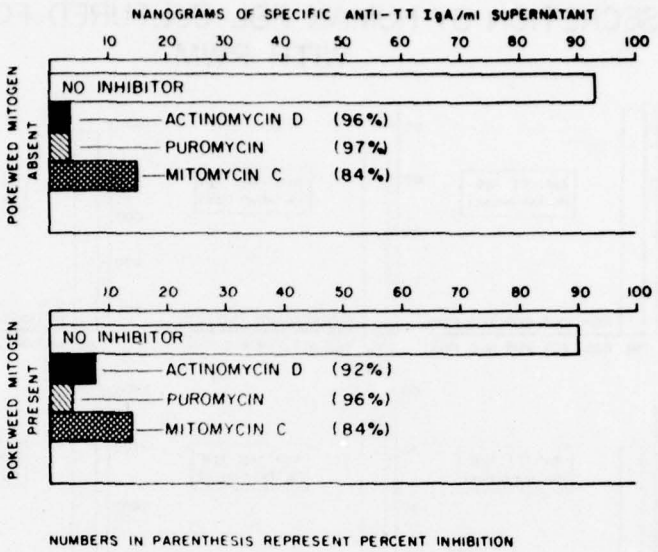
ABBREVIATIONS AND KEY

PWM	POKEWEED MITOGEN
TT	TETANOUS TOXOID
Mgc	MENINGOCOCCAL
Csss	GROUP C Mgc POLYSACCHARIDE

NL	□	NO INHIBITOR
RAD	▨	IRRADIATION (2000 R)
ACT	■	ACTINOMYCIN-D (10 μg/ml)
PUR	▥	PUROMYCIN (5×10^{-4} M)
M-C	▩	MITOMYCIN-C (25 μg/ml)
PHA	▧	PHA-P (1 μg/ml)

Figure 9

THE EFFECT OF INHIBITORS OF ANTIBODY SYNTHESIS ON THE PRODUCTION OF SPECIFIC ANTI-TT IgA BY CELLS CULTURED FOR 7 DAYS WITH OR WITHOUT PWM



NUMBERS IN PARENTHESIS REPRESENT PERCENT INHIBITION

THE EFFECT OF INHIBITORS OF ANTIBODY SYNTHESIS ON Ig SECRETION BY HUMAN PBL CULTURED FOR 7 DAYS WITHOUT PWM

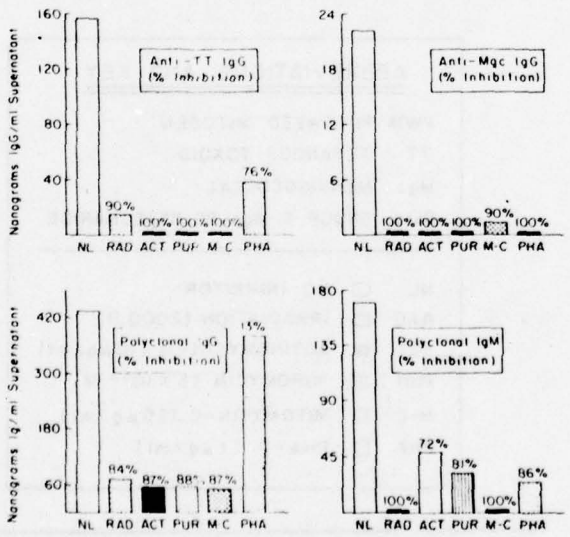
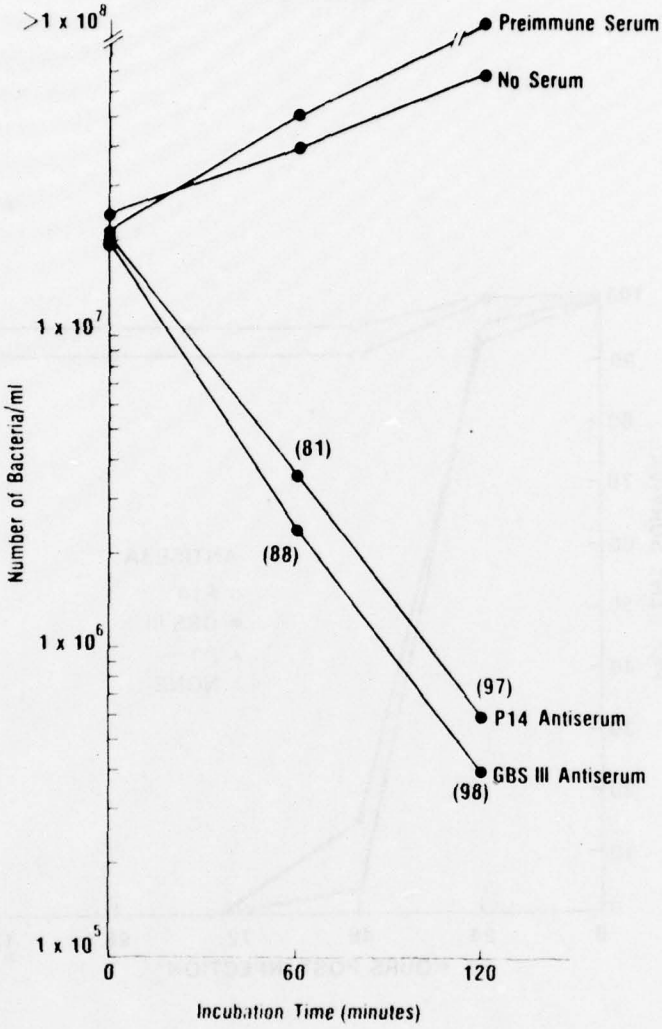
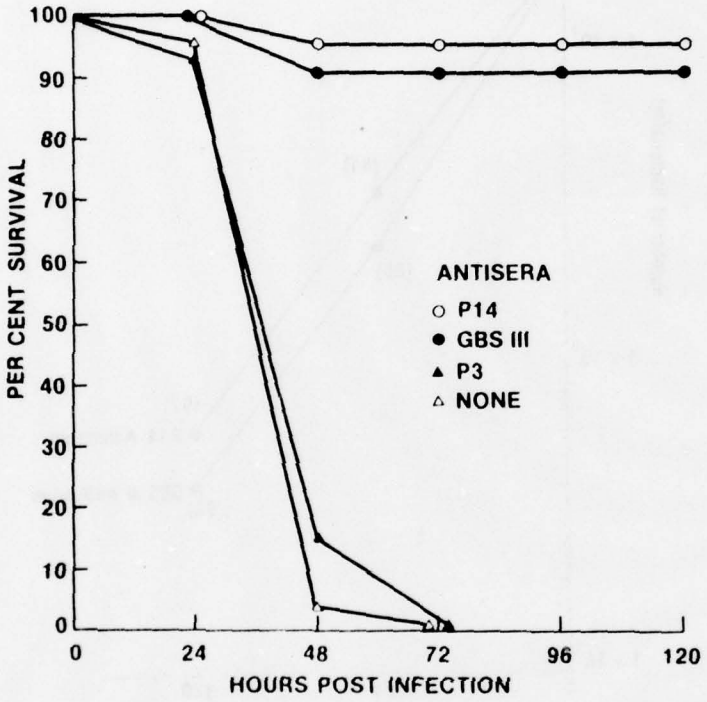


Figure 10



Effect of Rabbit Antisera on Group B Streptococci Type III in a (Pmn) Neutrophile Bactericidal Assay. No serum given (None); Serum obtained prior to immunization (Preimmune); Antisera directed against Group B streptococci type III (GBSIII); Antisera directed against S. pneumoniae type 14 (P14).

Figure 11



Effect of Type Specific Antisera on the Survival of Sucling Rats With Experimental Type III Group B Streptococcal Sepsis. Antisera directed against S. pneumoniae type 14 (P14); Antisera directed against Group B streptococci type III (GBSIII); Antisera directed against S. pneumoniae type 3 (P3); No serum given (None).

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 132 Bacterial Diseases of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6531	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	133
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Ecology of Plague

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS.	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 78	2	180
FISCAL YEAR — CURRENT 79	2	188

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.
24. (U) Using standard methods, specimens and sera from humans and animals are tested for the presence of Y. pestis and F-1 antibody to Y. pestis.
25. (U) 77 10 - 78 09 A correlation between the serological response to F-1 antigen and resistance to acute plague in rats has been established. Rats having IHA titers of 1/60 have a 50 percent chance of survival to subcutaneous challenge and a titer of 1/200 affords a 50 percent chance of survival in intranasal challenge with plague bacilli in fully virulent form. However, as time passed, the surviving vaccinated rats (17 percent of those vaccinated with living attenuated vaccines -- 26 percent of those vaccinated with killed USP vaccine -- 5 percent of those given passive immunization) developed chronic plague. ELISA techniques have been successfully utilized with F-1 antigen and use of other specific Y. pestis antigens to reflect plague infection with aberrant bacilli is under study. The production of a bacteriocin-like substance produced by all types of Y. pseudotuberculosis and the pesticin I of Y. pestis, which is inhibitory to the growth of type I Y. pseudotuberculosis, was greatly influenced by pH and various media constituents. Propagation of rough phage Ffm in Y. pestis strain DF-1 greatly restricted the affinity of Ffm for its normal host S. typhimurium, an effect not seen with Y. pestis strain Exu 1. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 133 Ecology of Plague

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Description.

To determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.

Progress.

The plague outbreaks of 1975 and 1976 in the United States have continued into 1977, the human cases occurring in areas previously described (Annual Report, WRAIR, 1977). Fort Wingate, New Mexico and the Pueblo Army Depot in Colorado, however, have again experienced plague epizootics. All plague strains isolated from plague patients over the past few years have been of fully virulent phenotype and sensitive to streptomycin and tetracycline.

Research during the period covered by this report has been directed towards: 1) testing the abilities of various vaccines and drugs to prevent acute and cryptic plague in rats; 2) developing and testing methodologies directly applicable to studies of Yersinia pestis phenotypes and chronic disease, and 3) improving diagnostic procedures for diagnosis and surveillance of plague infection. A study on the susceptibility of the California ground squirrel Spermophilus beecheyi was also completed. These matters form the basis of this report.

I. Studies with plague vaccines.

The relationship of F1 antibody titer to protection against plague was investigated by subjecting vaccinated, seropositive laboratory rats to virulent challenge and observing for survival. The indirect HA (IHA) test was employed for serology. Three groups of seropositive rats, with appropriate controls, were challenged sc. Rats in the first group had been vaccinated with 10^3 - 10^7 Y. pestis of the live avirulent strain EV76(51f) and were challenged either 26 days later with 5 X 10^5 Y. pestis 195/P or ten weeks later with 2 X 10^5 Y. pestis 195/P. The second group

consisted of rats that had received killed whole-organism plague vaccine U.S.P. (Cutter Laboratories, Berkeley, CA 94710, USA), with injections of either 1 ml on days 1, 32 and 49 followed by 0.2 ml on day 83 or 0.5 ml on days 1, 32, 49 followed by 0.1 ml on day 83. These rats were challenged 100 days after vaccination with 10^3, 10^4, or 10^5 Y. pestis 195/P. The third group contained rats vaccinated with F1 antigen (Walter Reed Lot 4, July 71) in Freund's complete adjuvant (Difco, Detroit, MI 48232, USA). The rats received an initial sc injection of 500 mcg F1 followed by booster injections of 200 mcg F1 at seven and 14 days. These rats were challenged six weeks later with 3.5×10^3 Y. pestis 195/P.

Two groups of rats were challenged concurrently by nasal instillation of 8.9×10^4 phagocytosis-resistant Y. pestis 195/P. One group consisted of rats vaccinated with F1 antigen, as outlined above, six weeks earlier. The other group contained rats that had been inoculated sc with 48 virulent Y. pestis 195/P and, two days later, administered an intraperitoneal injection of antiplague serum-globulin (Lederle Laboratories, Pearl River, NY 10965, USA) equivalent to 15 ml of rabbit serum. Rats surviving sc infection without discernible buboes were challenged intranasally after six months when it could be reasonably assumed, from previous studies (1), that the antiserum administered had been eliminated and any titer present was derived from active immunization. Challenge infections were done by subcutaneous (sc) inoculation or nasal instillation of virulent Y. pestis of the 195/P strain. Plague bacilli inoculated sc were cultured at 25°C to obtain phagocytosis-sensitive Y. pestis similar to those transmitted by fleas, whereas intranasal challenge employed encapsulated phagocytosis-resistant plague bacilli grown in vivo to simulate organisms implicated in inter-human transmission of pneumonic plague (2). Intranasal challenge employed Y. pestis 195/P taken from the peritoneum of a moribund WR rat.

Whereas unvaccinated rats always died after challenge infection, many vaccinated rats survived sc challenges with phagocytosis-sensitive Y. pestis or intranasal challenge with phagocytosis-resistant Y. pestis. Survival among vaccinated rats was in direct proportion to the titer of F1 antibody present at the time of challenge (Table 1). In vaccinated rats that died, death was delayed and the average time to death was directly correlated with the F1 antibody titer at time of challenge.

Patterns of survival following sc challenge were similar in groups of rats vaccinated with avirulent live vaccine, whole-organism killed vaccine or F1 subunit vaccine. Overall survival

Table 1. Experiments with laboratory rats.

Method of challenge	Treatment before challenge	Fl antibody titer at challenge[a]	Percent survival (Survivors/total)	Average day of death
Inoculation sc of 1 X 10^3 to 5 X 10^5 Y. pestis 195/P[c]	Vaccinated with live avirulent Y. pestis strain EV 76(51f)	None[b]	0 (0/23)	2.8
		≤1:16	0 (0/24)	4.2
		1:32-1:64	46 (6/13)	5.4
		1:128-1:256	91 (10/11)	6
		1:512-1:1024	100 (6/6)	-
	Vaccinated with killed whole-organisms plague vaccine USP	None[b]	0 (0/6)	3.2
		≤1:16	9 (6/70)	4.3
		1:32-1:64	50 (3/6)	13.3
		1:128-1:256	89 (8/9)	10
		1:512-1:1024	80 (4/5)	13
	Vaccinated with the Fl antigen of Y. pestis	None[b]	0 (0/5)	4.2
		1:32-1:64	40 (2/5)	9.0
		1:512:1:1024	97 (34/35)	11
Nasal instillation of 8.9 X 10^4 Y. pestis 195/P[d]		None[b]	0 (0/7)	3.0
	Vaccinated with the Fl antigen of Y. pestis	1:32-1:64	0 (0/4)	9.2
		1:128-1:256	50 (1/2)	13
		1:512-1:1024	100 (13/13)	-
	Infected with virulent Y. pestis 195/P and treated with antiserum two days later. Challenged 6 months post convalescence.	≤1:16	0 (0/3)	3.7
		1:32-1:64	0 (0/4)	6.2
		1:128-1:256	100 (5/5)	-
		1:512-1:1024	100 (4/4)	-

[a] Final serum dilutions in IHA test.

[b] Unvaccinated controls.

[c] Simulated flea challenge with phagocytosis-sensitive Y. pestis.

[d] Simulated pneumonic challenge with phagocytosis-resistant Y. pestis.

in rats challenged sc was 6% (6/94) at F1 antibody titers $\leq$1:16, 46% (11/24) at titers of 1:32-1:64, 90% (18/20) at titers of 1:128-1:256, and 96% (44/46) at titers of 1:512-1:1024.

Patterns of survival after intranasal challenge were similar in groups of rats that had derived F1 antibody titers from vaccination with F1 antigen or from a previous infection with virulent Y. pestis. Unlike the sc challenge, however, rats with titers $\leq$ 1:64 never survived intranasal challenge, reflecting the high virulence of phagocytosis-resistant Y. pestis. An increased mortality was not evident in rats possessing higher levels of antibody at the time of challenge. Overall survival from intranasal challenge was 86% (6/7) in rats with F1 antibody titers of 1:128-1:256 and 100% (17/17) at titers of 1:512-1:1024.

The results of our studies with laboratory Norway rats suggest that the HA titer of F1 antibody is a significant indicator of protection from acute bubonic plague in this species. In fact, retrospective analyses of published data for bubonic plague in wild-caught Norway rats, and also in laboratory monkeys, showed strikingly similar results. In all cases, greater numbers of animals survived experimental infections where the levels of F1 antibody present at the time of challenge were higher. In Norway rats and in monkeys, the majority of individuals possessing final F1 antibody titers $\geq$ 1:128 (e.g., initial serum dilutions $\geq$ 1:64 in the IHA test) survived severe challenges with plague bacilli of classical virulence. Some protection was evident even in vaccinated animals that succumbed to experimental plague, including individuals that had no measurable titer at challenge, as death was delayed. Possibly very small amounts of antibody to the F1 antigen were present that were not detected by the serological method used, or perhaps other immunological mechanisms had functioned for a time to delay death. Alternatively, some protection against virulent challenge may have been derived from exposure to other antigens of the plague bacillus. Similarly, a good correlation between F1 antibody titer and survival was found from our experiments with intranasal challenge of laboratory rats. Meyer (3) estimated that only 10% of the bacilli administered intranasally reach deeper respiratory passages (e.g., 8,900 Y. pestis in the present study), and infection of larynx, tonsils or other portions of the upper respiratory tract may also result. Although intranasal challenge is not as reliable as other methods (e.g., Henderson apparatus) for inducing a relatively uncomplicated plague pneumonia, our data indicated intranasal challenge to be much more severe than sc challenge, with no survivors among intranasally challenged rats possessing F1 antibody titers less than 1:128. The experimental data suggest

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Method of challenge	Treatment before challenge	Fl antibody titer at challenge[a]	Percent survival (Survivors/total)	Average day of death
Inoculation sc of 1×10^3 to 5×10^5 *Y. pestis* 195/P[c]	Vaccinated with live avirulent *Y. pestis* strain EV 76(51f)	None[b]	0 (0/23)	2.8
		≤1:16	0 (0/24)	4.2
		1:32-1:64	46 (6/13)	5.4
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	Vaccinated with the Fl antigen of *Y. pestis*	1:32-1:64	0 (0/4)	9.2
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		1:512-1:1024	100 (13/13)	-
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that F1 antibody titer may be a good measure of immunity to pneumonic as well as bubonic plague.

Although anti-F1 HA titer in Norway rats is an indicator of protection against acute plague, there is good evidence that this relationship does not extend to protection from chronic plague in these rats (4). Indeed, F1 antibody may have some influence on the development of chronic disease (5). As reported previously (Annual Report, WRAIR, 1977), Norway rats vaccinated with either living attenuated plague vaccine (EV) or killed plague vaccine USP, as well as rats passively immunized with polyvalent antiplague serum survived acute disease induced by challenge with fully virulent Y. pestis of phenotype $F1^+$, VW^+, P^+, PCF^+. Many survivors of acute disease, however, subsequently developed chronic plague infection. Many diverse phenotypes of the plague bacillus were identified in chronic lesions but of chief concern was phenotype $F1^-$, VW^+, P^+, PCF^+. A 2.5 year study has been completed which provides information on the rates at which chronic disease occurs in vaccinated rats (Table 2) and the persistence of various phenotypes in chronic lesions (Table 3).

Several reports have shown virulent Y. pestis survive in the lesions of patients following treatment with effective antibiotics (Annual Report, WRAIR, 1977). Concern over the possibility of these surviving organisms establishing a chronic infection prompted a long range study where two groups of 20 normal R. norvegicus were treated with either streptomycin or tetracycline for 10 days after plague infection was well-established. Treatments were successful and chronic disease was not observed in these rats.

II. Experimental plague in the California ground squirrel.

Plague in the California ground squirrel, Spermophilus (Citellus) beecheyi, has presented a public health threat of considerable magnitude since 1908 and many human cases of plague have originated from contact with diseased squirrels. This study of squirrels collected from a defined locale with a previous history of epizootic plague was designed to examine the current potential for epizootics in this species and the role of California ground squirrels in maintaining enzootic plague foci. Young California ground squirrels were trapped on Fort Hunter Liggett in southeastern Monterey County, California, disinfested and acclimated to laboratory conditions in individual cages for two months before use. The squirrels adapted readily and exhibited a well-conditioned, healthy appearance and rapid growth.

Table 2. Rates for chronic plague in laboratory rats.

Vaccination or passive immunization	*Yersinia pestis* phenotype isolated	Incidence*
Living avirulent plague vaccine(EV)	$F1^-:VW^+:P^+:PCF^+$	17%(2/12)
Killed plague vaccine (USP)	$F1^+:VW^+:P^+:PCF^+$	26%(9/34)
Anti-plague serum (polyvalent)	$F1^-:VW^+:P^+:PCF^+$	4%(3/76) } 5%
	$F1^+:VW^+:P^+:PCF^+$	1%(1/76) }

* Rates for rats that survived acute challenge with typical *Y. pestis* as previously described (Annual Report, WRAIR, 1977).

Table 3. Persistence of plague infection in some Rattus norvegicus surviving challenge with classical plague organisms.

Immunization before challenge	Isolation of Yersinia pestis	
	Phenotype	Months post-challenge
Living avirulent plague vaccine (EV)	$F1^-:VW^+:P^+:PCF^+$	18
Killed plague vaccine (USP)	$F1^+:VW^+:P^+:PCF^+$	17
Anti-plague serum (polyvalent)	$F1^+:VW^+:P^+:PCF^+$	11
	$F1^-:VW^+:P^+:PCF^+$	24

Squirrels were bled two weeks prior to challenge and several times thereafter. The sera obtained were inactivated at 56°C for 30 minutes and tested (16) for indirect hemagglutination (HA) antibody to the specific fraction 1 (F1) antigen of the plague bacillus. Strain 761000 of Y. pestis was used for experimental infections. This strain was recovered from a fatal human case of plague that occurred during April, 1966, in Kern County, California, shortly after a die-off of ground squirrels. Squirrels were challenged by sc inoculation of Y. pestis cultured at 25°C to simulate infection by fleabite (2). All squirrels that died were autopsied, and isolation of Y. pestis was attempted from spleen and heart's blood.

F1 antibody was not detected in sera taken from California ground squirrels before initial challenge. Confirmed plague deaths occurred at all levels of infection, including one squirrel exposed to only 6 Y. pestis (Table 4). The LD_{50} for ground squirrels equalled 6,070 Y. pestis, equivalent to 567 mouse LD_{50}. Squirrel mortality, as well as serological response to infection, was dose related. Squirrels succumbing to plague lived an average of 7.9 days from challenge to death.

Of 26 squirrels surviving infections with $\leq$ 1 LD_{50}, 73% did not develop a titer of IHA antibody. However, all squirrels surviving $\geq$ 10 LD_{50} demonstrated seroconversion, although titers never exceeded 1:64 (Table 5). The ID_{50} for squirrels, calculated on the basis of plague death and seroconversion as indices of infection, equalled 256 Y. pestis.

Surviving squirrels were reinfected sc with 6,800 Y. pestis (544 mouse LD_{50} or 27 squirrel ID_{50}). With the exception of one squirrel that had developed a transient HA titer of 8 about 11 weeks after initial infection, these animals never exhibited titer prior to their second infection. Four squirrels died of plague and 14 survived. Four surviving squirrels produced low levels of antibody while nine remained seronegative. The squirrel that had demonstrated a titer of 8 some months before reinfection developed a titer of 1:256 four weeks thereafter (Table 5).

Although, in comparison with laboratory mice, S. beecheyi is relatively resistant to infection with Y. pestis, the resistance should be viewed from an ecological perspective. Fleas have been found to transmit up to 24,000 Y. pestis through feeding (6). Thus, the bite of a single infected flea could transmit about 4 LD_{50} (or 42 ID_{50}) of Y. pestis when feeding upon these squirrels.

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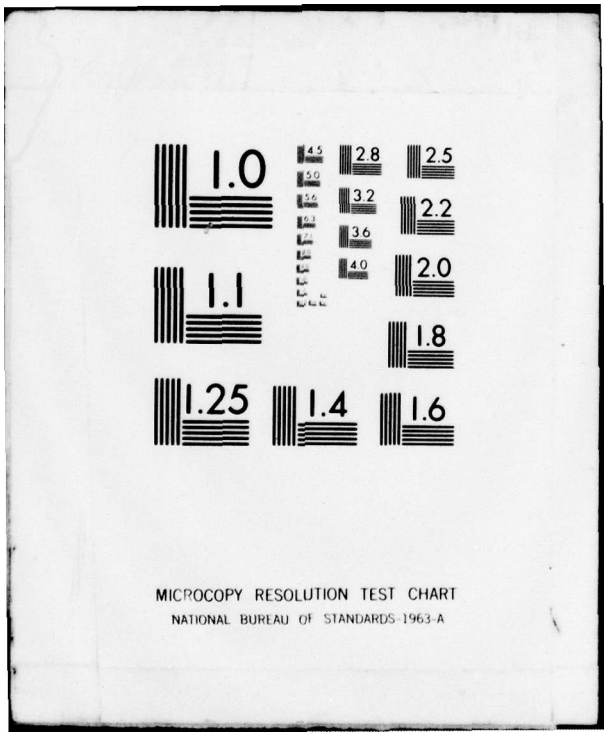

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Table 4. Susceptibility of California ground squirrels to infection with *Yersinia pestis*.

Number of *Y. pestis*[a] inoculated	Number dead/total[b]	Seroconversions/survivors	Number infected/total[c]
6	1/8 (13%)	1/7 (14%)	2/8 (25%)
61	2/10 (20%)	2/8 (25%)	4/10 (40%)
607	4/10 (40%)	1/6 (17%)	5/10 (50%)
6,070	3/8 (38%)	3/5 (60%)	6/8 (75%)
60,700	4/6 (67%)	2/2 (100%)	6/6 (100%)
607,000	2/4 (50%)	2/2 (100%)	4/4 (100%)
6,070,000	3/4 (75%)	1/1 (100%)	4/4 (100%)

[a] The squirrel LD_{50} was 6,070 *Y. pestis*, equivalent to 567 mouse LD_{50} (one mouse LD_{50} was 10.7 *Y. pestis*).

[b] Deaths were confirmed as plague by isolation of *Y. pestis*. Young squirrels in the various groups were picked at random and without regard to sex.

[c] Number infected (deaths plus seroconversions) indicates an ID_{50} of 256 *Y. pestis*.

Table 5. Serological response of surviving California ground squirrels to infection or reinfection with Yersinia pestis.

F1 antibody titer	Number of ground squirrels with indicated IHA titer	
	Initial infection	Reinfection
---*	19	13
1:4		
1:8	5	1
1:16	2	2
1:32	2	1
1:64	3	
1:128		
1:256		1

* Initial serum dilution tested was 1:4.

The data for infective doses up to 6,800 Y. pestis probably reflect best the consequences of natural plague infection by fleabite. These data indicate that at least 70% of the squirrel population sampled would become infected from the bite of a single infective flea, although only about 39% would be expected to die. Squirrels that survive plague certainly could support infected fleas, and subsequent infections of susceptible squirrels, recruited into the population by immigration or natality, would serve to perpetuate disease resulting in an enzootic plague focus.

Epizootics may still occur among relatively plague resistant squirrels. Occasional "die-offs" due to plague have occurred in areas where a history of past epizootics would suggest that resistance to plague should have developed. Indeed, the experimental data from this and other studies (7) indicate that mortality increases with the frequency of reinfection. Approximately 20% of reinfected squirrels succumb to disease, with bacteremia sufficient to infect fleas. Furthermore, as mortality increases in a squirrel population, the flea load, which under normal circumstances may average over 60 fleas per animal, increases on surviving squirrels enhancing the probability of reinfection by fleabite. We have observed flea indices in excess of 230 fleas per squirrel on survivors of field rodenticide campaigns (8). Obviously, numerous inoculations of plague bacilli can occur over a short period of time under similar circumstances when infected fleas are present. Subsequent squirrel mortality might be dose related; mortality increased to an average of 64% at doses $\geq$ 10 LD_{50} in our laboratory experiments, and a single natural inoculation by fleabite may represent up to four squirrel LD_{50}. In addition, reinfections by contact with moribund animals, many of which exhibit bacteremia with bloody noses, or from the cannibalism of infected carcasses must be considered. The titers of HA antibody we observed in California ground squirrels help to explain why seropositive squirrels have been seldom detected in serosurveys conducted during enzootic periods. Not only are there few infective fleas to infect squirrels, but squirrels surviving an infection from the bite of a flea probably demonstrate only low levels of F1 antibody, and, in serosurveys, only indirect HA titers $\geq$ 32 are generally accepted as diagnostic evidence of plague infection. During and shortly following epizootics, when infective fleas are more abundant, reinfections of squirrels may stimulate increased levels of antibody; i.e., a serological response of 8 resulting from an initial, non-lethal infection was increased in our study to 256 after a subsequent inoculation of Y. pestis. Consequently, seropositive squirrels could become more numerous at such times. Significantly, recent experiments with rock squirrels (Spermophilus variegatus) collected in New Mexico have demonstrated that these animals, like California ground squirrels, frequently survive initial plague infection and produce

only low levels of HA antibody (9). In effect, the results reported in this study may reflect the serological responses to infection exhibited by other plague resistant rodents.

III. Cooperative study on tests for the detection of plague antibodies.

Recent work compared World Health Organization (WHO) reagents and procedures for the indirect hemagglutination (IHA) test for F1 antibody with the reagents and procedures utilized for IHA at WRAIR. The WHO supplied reagents (manufactured in the USSR), detailed protocols for the WHO test and 18 lyophilized, unknown sera for testing. The sera were rehydrated and then tested simultaneously with the WHO reagents by the WHO test procedure and with WRAIR reagents and methods.

Titers for seven sera were < 1:40 in both WHO and WRAIR tests, and three sera demonstrated titers $\geq$ 5,120 by both tests. Eight sera gave titers of 1:80 - 1:320 (GMT = 1:160) by the WRAIR test and 1:20 - 1:160 (GMT = 1:64) by the WHO test. Thus, the average difference between tests slightly exceeded one two-fold dilution of serum. Subsequent tests on control sera demonstrated that this small difference between tests may have resulted from a somewhat heavier concentration of fraction-1 sensitized cells employed in the WHO test which decreases test sensitivity.

Results of these tests (titers from WHO IHA tests averaged 40% of titers from WRAIR IHA tests) were very similar to the results from a previous study that compared the WHO hemagglutination-inhibition (HAI) test with WRAIR's IHA test (10) where titers from WHO HAI tests averaged 56% of titers from WRAIR IHA tests.

IV. Studies with diagnostic reagents.

Although the indirect HA test has been utilized in plague serology for over 20 years, recent observations on cryptic plague (Annual Report, WRAIR, 1977) have indicated a requirement for serological procedures that employ specific antigens, other than F1, of the plague bacillus. These tests are especially desirable to detect disease resulting from infections with Y. pestis of phenotype $F1^-$, V/W^+, P^+, PCF^+, occasionally found in chronic infections (see above). The ELISA technique was selected for evaluation as representing a system readily applicable to the study of both a multiplicity of antigen-antibody reactions and several other problem areas of routine plague serology.

Exploratory studies were conducted using the methodology of Ruitenberg et al. (11). Various plague antisera prepared in rabbits and albino Rattus norvegicus and several clinical specimens of acute and convalescent sera obtained from either plague patients or their contacts were tested in special ELISA plates (Microbiological Associates, Bethesda, Md.) coated with F1 antigen (WRAIR Lot 4A). Horseradish peroxidase conjugated antibody antigen reactions were detected with 5-amino salycylic acid: horseradish conjugated anti-rabbit IgG and anti-human IgG and IgM antisera were prepared in goats; anti-rat IgG was prepared in rabbits; all were purchased from Miles Laboratories. Enzymatic activity was measured in a spectrophotometer at 455 mμ. Endpoints of the titration were derived by comparing the results obtained in the test series per se with those of normal serum "background" controls and with those obtained by testing individual sera in an additional control titration free of F1 antigen.

Initial checkerboard titrations showed the optimum concentration of F1 for coating to be 10 μg/ml. Use of the purified F1 antigen and the horseradish peroxidase conjugate provided a remarkably clean and reproducible test with all antisera tested. The slopes of all reactions were directly linear showing a definite effect of careful dilution of antibody regardless of animal species tested. Further, although the results of tests in this study were read in a spectrophotometer, the stronger titers could be easily read with the naked eye.

Indirect hemagglutination tests were conducted on the various sera in-tandem with ELISA tests. These results indicated that the two tests were in good agreement as to ability to test the presence or absence of F1 antibody although the ELISA test was, in some instances, more sensitive than the IHA tests as performed and interpreted in this laboratory (Table 6).

Some 11 pairs of acute and convalescent sera obtained from bacteriologically confirmed cases of bubonic plague were available. Unfortunately, data on disease onset and on which dates the sera were drawn were not provided with the specimens. These paired sera were, however, tested in the ELISA system to detect either IgG or IgM. The results (Table 7) showed that both IHA and ELISA tests are adequate for the detection of F1 antibody, although, as stated above, the ELISA test was both more sensitive and, perhaps more effective in detecting antibody in the acute specimen. Also, the ELISA method was capable of conveniently demonstrating the presence of IgM. Unfortunately, the data did not permit an estimate of the time-frame involved in the appearance or disappearance of IgM. In another series of sera

Table 6. Comparison of ELISA and IHA tests for F1 antibody: Results expressed as group geometric mean titers.

Test sera	Number tested	Method		
			ELISA	
		IHA GMT	GMT	$\bar{X}$ slope
Rabbit	5	1:4681	1:7021	$.014 \pm .019$
R. norvegicus	11	1:658	1:329	$.099 \pm .07$
Man	45	1:64	1:512	$.071 \pm .08$

Table 7. Titers of F1 antibody is acute and convalescent sera collected from 11 bacteriologically confirmed cases of bubonic plague.

Test method	Acute Specimens* GMT	Convalescent Specimens* GMT
IHA	1:4	1:384
ELISA		
a) IgG	1:91	1:3803
b) IgM	1:16	1:1609

* Dates for onset of disease and serum collections not available.

collected from 15 patient contacts in an unproven (although highly probable) outbreak of plague pneumonia F1 antibody (IHA 1:32 : IgG 1:598 : IgM 1:135) was demonstrated in one specimen).

Although the true usefulness of the ELISA technique awaits further evaluation, initial results are encouraging. With the relative purity of Y. pestis antigens available, i.e., F1, murine toxin, L, V/W, and the general ease and reproducibility afforded by the test system, the ELISA tests should be of extreme value in the laboratory and in the field.

V. Bacteriological studies.

In collaboration with the Plague Branch, CDC, USPHS, 18 strains of Y. pestis isolated from plague cases occurring in the United States in 1977 were examined for virulence determinants and antibiotic susceptibilities. Without exception, these strains of Y. pestis possessed all the virulence determinants characteristic of classical plague bacilli and were completely susceptible to streptomycin and tetracycline.

VI. Plague bacteriophages.

Studies on plague bacteriophages have been expanded to include the rough specific bacteriophages of the Enterobacteriacae (Annual Report, WRAIR, 1977). Three of these bacteriophages Br_2, 6SR and BR60 tested on Y. pestis lysed all strains tested at 19C and 22C; demonstrated some lysis at 28C and none at 37C. Phage Ffm only lysed Y. pestis strain DF1 ($F1^{-}VW^{+}P^{+}PCF^{+}$) and this lysis occurred only after 5 days incubation at 19C and 22C. Phage Ffm propagated in Y. pestis DF1 or freshly propagated in Salmonella typhimurium, lysed all strains of Y. pestis tested. However, when propagated in the non-encapsulated virulent Y. pestis strain M23 at 25C, the resulting phage appeared to distinguish pigmented from non-pigmented Y. pestis. Lysis of pigmented strains was not observed at any temperature after 18 hr incubation. Lysis of non-pigmented strains, however, was always observed. Rough phages ES18 and C21, have not, as yet, demonstrated any effects on Y. pestis. Studies on these phages are being continued, however, in the hope that by manipulations of host, plaque selection and propagation temperature, systems to detect various virulence factors of the plague bacillus much more rapidly than is currently possible may be developed.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 133 Ecology of Plague

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Publications:

1. Williams, J.E., Harrison, D.N., Quan, T.J., Mullins, J.L., Barnes, A.M., and Cavanaugh, D.C. Atypical plague bacilli isolated from rodents, fleas, and man. Amer. J. Public Hlth. 68: 262-264, 1978.

Presentations:

1. Cavanaugh, D.C. Ecology of plague. Presented at the Baltimore Round Table of American Military History, Baltimore, Md., 16 March 1978.

2. Williams, James E. The ecology of plague. Presented at a seminar at the Institute of Ecology, University of Georgia, Athens, Ga., 12 May 1978.

3. Williams, James E. Plague. Presented at the Parasitology Seminar, Armed Forces Institute of Pathology, WRAMC, 19 May 1978.

4. Cavanaugh, D.C. Plague. Presented at the Tropical Medicine Course, WRAIR, 17 July - 25 August, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0A 6449	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	134
b. CONTRIBUTING				
c. XXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Immunological Mechanisms in Microbial Infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003400 Clinical Medicine

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62 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

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c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	5.0	211
	CURRENT 79	2.0	182

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21. GENERAL USE

Foreign Intelligence not considered

ASSOCIATE INVESTIGATORS

NAME:

NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunity; (U) Antibodies; (U) Infectious Diseases; (U) Complement Fixation; (U) Radioimmunoassay; (U) Serodiagnosis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of this work unit is to elucidate the mechanisms operative in the natural and artificial induction of immunity to a variety of microbial infections of military importance. This includes the study of infections in model systems and the development of methodologies for the study of the immune reaction in humans for research as well as diagnostic evaluations.

24 (U) The approaches used for these studies involve the measurement of various parameters of disease and of the immune response to disease in both in vivo and in vitro experiments. A variety of diseases are attacked. Immunological phenomena common to a variety of different diseases are also studied.

25 (U) 77 10-78 09 The chemotherapy of leishmaniasis has been shown to be markedly enhanced through the use of liposomes as drug carriers. In this system glucantime, a lipid soluble agent, is incorporated into liposomes injected into animals with leishmaniasis. The liposomes are cleared selectively from theblood by macrophages, the cells which also contain the leishmania parasites. The phagocytized liposomes are thus delivered to the leishmania. This delivery of drug to parasites in concentrated form allows cure of leishmaniasis with a several hundred fold lower dose of the drug than when liposomes are not used. In another area, immunochemical studies of African trypanosome antigens have revealed that some methods currently employed for isolation may result in cleavage of the antigen, presumably through proteolytic enzymes. Methods developed in this laboratory result in less serious degradation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 1977 - 30 Sep 1978.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 134 Immunological Mechanisms in microbial Infections

Investigators:

Principal: LTC Carl Alving, MC, M.D.; Roberta Richards, Ph.D.; John Barbaro, Ph.D.; Donald T.O. Wong, Ph.D.

Assistant: SP5 Katharine Urban, M.S., B.S.; SP6 Barry Ellis, B.S.

1. Impact of lipid A from endotoxin on endothelium.

An intact physiologic endothelium is the first line of defense against noxious agents and conditions that may promote vessel wall damage. Bacterial endotoxins are substances reported, although inconsistently to produce vascular lesions. According to our SEM study, lipid A, the biologically active form of endotoxin, has direct influence on endothelial cells. Lipid A, 0.005 to 1.0 μg/ml Dulbecco's phosphate buffered saline, was perfused in the fresh human umbilical vein model. Companion controls from the same umbilical cord were run concurrently. The thromboresistive or thrombogenic capabilities of the intact vessel wall components were tested by perfusing washed apyrase treated autologous platelets. Lipid A, from Shigella flexneri, elicited endothelial cell injury, the quality and magnitude of which was concentration dependent and seemed more extreme in the presence of platelet poor plasma. Signs of endothelial cell stress ranged from (1) increased microvillus activity through broad bleb formation to minute membrane lesions and (2) from tiny gaps in cell border junctions to partial or complete detachment from subendothelium. The amount of exposed subendothelium was quantified by morphometry. In contrast to normal endothelium, lipid A injured endothelium was thrombogenic (attractive for platelet adhesion). Subendothelium exposed by endothelial cell detachment permitted platelet adhesion and various degrees of activation. We conclude that isolated lipid A of bacterial endotoxin is competent alone to provoke vascular injury that may lead to thrombotic or other sequalae.

2. Interactions of C-reactive protein and complement with liposomes

Interactions between C-reactive protein (CRP) and liposomal model membranes containing phosphatidylchoine were investigated. These interactions, in the presence of human serum, resulted in consumption of each of the components of the classical complement pathway (C1-C9) and also resulted in complement-dependent damage and release of trapped glucose from certain types of liposomes. CRP-initiated lysis of liposomes was strongly dependent upon membrane lipid compositon. Optimal activity occurred with positively charged liposomes containing galactosylceramide (galactocerebroside); positively charged liposomes lacking galactocerebroside released much less glucose, while negatively charged liposomes, either with or without galactocerebroside, did not release glucose at all. Glucose release was inhibited by free phosphocholine. Lesser, but significant, "background" glucose release independent of the presence of CRP also was observed with positively charged liposomes containing galactocerebroside, and this was associated with marked preferential consumption of the later-acting complement components (C3-C9). C2-deficient human serum failed to support CRP-dependent glucose release, but glucose release was observed upon reconstitution was observed upon reconstitution of the serum with C2. Guinea pig complement also did not support CRP-mediated glucose release, but upon addition of human Clq substantial glucose release was observed. We conclude that (I) CRP can sensitize appropriate liposomes for complement-dependent damage via the primary complement pathway starting at the level of Clq; (II) of those studied, liposomes that are most susceptible to membrane damage contain phosphatidylcholine, have a positive charge, and contains a ceramide glycolipid; and (III) such liposomes also are sensitive, although to a much lesser degree, to complement-dependent lysis initiated in the absence of CRP and involving consumption of terminal in excess of early acting complement components.

3. Therapy of leishmaniasis: Superior efficacies of liposome-encapsulated drugs.

Liposomes containing antimonial compounds trapped in the aqueous phase were tested in the treatment of experimental leishmaniasis. The rationale of this approach is based on the hypothesis that the liposomes and the parasite are taken up by the same cell, the reticuloendothelial cell, and we present electron microscopic evidence that supports this hypothesis. Suppression of leishmaniasis was quantified by determining the total number of parasites per liver from impression smears. When two

antimonials, meglumine antimoniate and sodium stibogluconate, were encapsulated within liposomes, each was more than 700 times more active compared to either of the free (unencapsulated) drugs (Fig. 1). After infection, if untreated, all of the hamsters eventually would die from the disease. Liposome-encapsulated meglumine antimoniate was about 330-640 times more effective in causing a drop in the death rate than was the free antimonial. The efficacy of treatment was influenced by the lipid composition and charge of the liposomes. For example, positively charged liposomes containing egg phosphatidylcholine were much less effective than negatively charged ones. In contrast, positively and negatively charged sphingomyelin liposomes were equally effective. Liposomes containing phosphatidylserine (which were negatively charged, but also had a much higher charge density) were among the less-effective preparations. Among those tested, the most consistently efficacious liposomes contained highly saturated long-chain phospholipids (e.g., dipalmitoyl phosphatidylcholine), cholesterol, and a negative charge.

We conclude that liposomes may be useful as carriers of drugs to treat infectious disease involving the reticuloendothelial system. The toxicities of antimony are very similar to those of arsenic. Encapsulation of antimonial drugs and reduction of the dose required for effective therapy should minimize such systemic toxicities as acute cardiomyopathy and toxic nephritis.

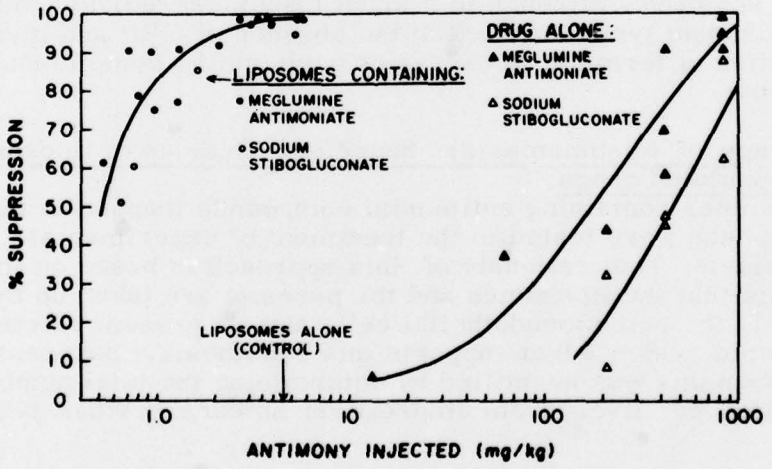


Fig. 1. Suppression of Leishmania by liposomes encapsulated

antimonials. Liposomes consisted of DMPC/Chol/DCP (molar ratio, 2/1.5/0.22). Treatment of the hamsters (11 animals for each point) was begun 17 days after infection and one-fourth of the indicated doses were injected intracardially daily for 4 days. The surviving animals were sacrificed 32 days after start of therapy. Control groups also were injected with equivalent volumes of liposomes swollen in 0.15 M NaCl. ● Liposomes containing meglumine antimoniate; ○ , liposomes containing sodium stibogluconate; ▲ , meglumine antimoniate alone; △ , sodium stibogluconate alone.

4. Investigation of Immunologic Imbalance in Atopic Dermatitis.

A. Objective. The purpose of this study is to determine the presence of a possible immunologic imbalance in atopic dermatitis, particularly in regard to suppressor T-cell function. Hopefully, this study will provide a rational modification of present day therapy in addition to providing valuable insight into basic immunologic mechanisms.

B. Description: Atopic dermatitis is a common, incapacitating, dermatological condition, but its cause remains obscure. Infection, immunological dysfunction as well as hereditary, emotional stress, B-adrenergic blockage and environment have all been implicated. In recent years, considrable attention has been focused on the immunological abnormalities associated with atopic dermatitis. Atopic dermatitis occurs commonly in certain immunological deficiency diseases such as sex linked agammaglobulnemia, ataxia telangiectasia, and Wiskott Aldrich syndrome. In addition, a number of clinical observation have been made, which when considered as a group, suggest a possible derangement of cell mediated immunity. Impaired cellular immunity is also suggested by certain in vitro studies. There have been several reports of decreased number of circulating T-cells as well as reports of decreased lymphocyte transformation with various polyclonal mitogens. It has been demonstrated in experimental animals that a certain subpopulation of T-cells (suppressor T-cells) modulates B-cell function (antibody production) particulary in regard to IgE. It is not known whether there is a similar subpopulation of T-cells in man which serve a similar regulatory function over IgE antibody. Most patients with atopic dermatitis usually have increased serum IgE levels, the degree of elevation corresponding roughly to the extent and severity of the skin disease. The purpose of this study is to determine if a similar

subpopulation of T-cells acts in a regulatory function over IgE antibody production in man. Rabbit anti ox erythrocytes (ox RBC) serum pool was concentrated with ammonium sulfate prior to chromatographic separation on DE-52, equilibrated with 0.1M TRIS, pH 7.9 for the preparation of IgG anti ox RBC. The breakthrough peak, representing pure IgG, was pooled and concentrated. Although the material represented only 15% of of the total globulin added to the column, it contained no IgM.

A second pool of rabbit anti ox RBC serum, treated, in a similar manner, was separated on G-200 Sephadex equilibrated with 0.05M TRIS, pH 8.0 for the isolation of IgM anti ox RBC reagent. Only the ascending portions of the macroglobulin peak were pooled and concentrated. This material represented approximately 5% of the protein added to the column and was found to be monospecific.

T-cells were purified (isolated) by treating a mononuclear cell-rich population with sheep erythrocytes and centrifugation of the resulting rosetted cells over ficoll-hypaque gradient. The pelleted T-cells, free of erythrocytes by hypotonic lysis, were then examined for Fc receptors of IgG and IgM using a rosetting technique with IgM anti ox RBC or IgG anti ox RBC complexes (ox RBC coated with specific antibody).

Progress:

Of crucial importance in studies involving separation subpopulation of T-cells is the purity of the reagents. The purity of the IgM and IgG anti ox RBC sera was tested by Ouchterlony and immuno-electrophoresis using many different preparations of polyclonal and monoclonal anti rabbit immunoglobulins.

The IgG preparation contained no IgM material and the IgM preparation contained no IgG material. An additional criterion for the purity of IgM anti ox RBC was its susceptibility to 2 mercapto-ethanol (2ME). The data from microtiter hemagglutination assay with enzyme treated on RBC showed IgM anti ox RBC, prior to treatment with 2 ME, had a titer of 1/2560; whereas, after 2 ME teatment the titer was less than 1/20. This represents at least a 99% decrease in agglutination, which clearly demonstrates the purity of the IgM preparation.

Once the purity and optimal concentration of the reagents were established, it was possible to determine if there was indeed a correlation between T-cells and serum IgE levels and more importantly what subpopulation of T-cells are involved. Two groups were compared, based primarily on the level of serum IgE. Normal individuals with serum IgE levels of less than 500 I.U./ml were matched by age, sex and race with atopic dermatitis patients with serum IgE levels greater than 1000 I.U./ml. Table 1 lists the percentage of T-cells population with receptors for Fc of IgG (TG) and IgM (TM). There is little or no difference between the percentage of TM subpopulation of normal individuals and atopic dermatitis patients. In contrast, there is a significant difference between the percentage of TG subpopulation of normal individual and atopic dermatitis patients. Of the seven patients tested, only one had the normal percentage of TG cells, while the remainding six showed at least a 70% decrease in TG cells.

Discussion:

The preliminary results clearly demonstrate a decrease in the percentage of TG cells in patients with atopic dermatitis in contrast to normal individuals although the data suggest a correlation between high serum IgE levels and decreased number of TG cells, the data is not conclusive. Further experiments are planned to include additional controls - atopic individuals with elevated serum IgE with no evidence of dermatitis and patients with chronic dermatitis other than atopic dermatitis with low levels of serum IgE.

PERCENTAGE OF T-CELL POPULATION WITH RECEPTORS FOR Fc PORTION OF IgG (TG) AND IgM (TM).

Normal Health Individuals			Patients, Atopic Dermatitis		
Controls	TG Cells	TM Cells	Patients	TG Cells	TM Cells
1	10	55	A	0	66
2	10	54	B	10	58
3	12	53	C	3	40
4	12	69	D	4	47
4	12	69	E	4	68
5	10	54	F	2	50
5	10	54	G	1	58
Mean ± SG	10.9 ± 1.1	58.3 ± 7.3	Mean ± SD	3.4 ± 3.3	55. 3 ± 10.2

TABLE 1

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 134 Immunological Mechanisms in Microbial Infections.

Literature Cited.

Publications:

1. Alving, C.R., Richards, R. L., and Gewurz, H. Complement activation by liposome-CRP complexes: influence of membrane composition. J. Immunol. 120, 1761 (1978).

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8. Alving, C.R., Steck, E.A., Hanson, W.L., Loizeaux, P.S., Chapman, Jr., W.L. and Waits, V.B. Improved therapy of experimental leishmaniasis by use of a liposome-encapsulated antimonial drug. Life Sci. 22, 1021-1026 (1978).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6513	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102B S01	00	135
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Mechanisms of Transmission of Hepatitis Viruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	2.5	68
FISCAL YEAR CURRENT 79	2.5	87

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Viruses; (U) Hepatitis; (U) Antigen; (U) Immunology

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)

23 (U) To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses - antigens and antibodies to determine host factors important in resistance to disease and infection in military personnel.

24 (U) New methods for identification and antigenic analysis of hepatitis viruses are under development. The immune response of patients infected with hepatitis viruses is studied to define sensitive parameters of infection and to define factors critical in immunity. The epidemiology of hepatitis B in military populations is defined.

25 (U) 77 10 - 78 09 A sensitive and specific microtiter solid phase radioimmunoassay (RIA) for hepatitis A antigen (HAV) and antibody to HAV (anti-HAV) was developed utilizing antigen from stool of humans previously infected with the MS-1 strain of HAV and immune sera collected in Alaska in FY 77. Two chimpanzees were infected with HAV and have provided additional antigen and antibody. The RIA for anti-HAV was used to provide specific serodiagnosis during an outbreak of hepatitis at Schofield Barracks, Hawaii in June. Commercial lots of immune serum globulin were found to have titers of anti-HAV of 1/1000 - 1/3000. The specificity of an assay for antibody to hepatitis B core antigen was improved by partial purification of the antigen. The antiviral drugs ribovirin and adenine arabinoside were found ineffective in arresting hepatitis B surface antigen production in vitro by a human hepatoma cell line. The frequency of hepatitis A, B and other viruses causing hepatitis in military personnel will be determined. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 135 Mechanisms of Transmission of Hepatitis Viruses

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Associate: LTC J.C. Gaydos, MC; J.M. Dalrymple, Ph.D.; SSG T.E. Simms; SP5 C.D. Brown; SP4 J.R. Payne; H.G. Cannon

Description

To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibody to determine host factors important in resistance to disease and infection.

Progress

I. Hepatitis A virus (HAV).

A specific and sensitive radioimmunoassay for the detection of either HAV or antibody to HAV (anti-HAV) has been developed. A large amount of HAV antigen, for use in the radioimmunoassay, has been produced in chimpanzees. The radioimmunoassay has been successfully applied to a recent outbreak of hepatitis type A among troops at Schofield Barracks, Hawaii.

A. Development of a solid phase radioimmunoassay for HAV and anti-HAV antibodies.

1. Background

Several different techniques have been described for the detection of HAV and anti-HAV, including complement fixation, immune electron microscopy, immune adherence hemagglutination and solid phase radioimmunoassay (SPRIA). SPRIA and immune electron microscopy methods both offer a high degree of sensitivity as well as the ability to detect both IgM and IgG

classes of anti-HAV. Immune electron microscopy, however, is cumbersome and not suited to the study of large numbers of specimens. While the immune adherence hemagglutination assay is perhaps more sensitive than the radioimmunoassay in the detection of IgG anti-HAV, it fails to detect IgM antibody. The complement fixation assay is both insensitive and wasteful of reagents. For these reasons, SPRIA was chosen as the method for detection of HAV and serodiagnosis of hepatitis type A.

The radioimmunoassay is based largely on a previously reported enzyme-linked immunosorbant assay (ELISA) for hepatitis A (Mathiesen et al., 1978), but incorporates several important modifications. At present, the ELISA test offers no advantage over the radioimmunoassay, given the availability of gamma-radiation counting equipment. In the future, however, the development of automated ELISA plate readers may make conversion to an ELISA test advantageous. Such a conversion of methodology should be relatively simple.

The radioimmunoassay is carried out in 96 well microtiter plates and consists of a "sandwich" reaction (plate-antibody-antigen-^{125}I-antibody). Specificity of the reaction is determined by competitive inhibition of radiolabelled ^{125}I-antibody with non-labelled anti-HAV.

2. Materials

a. anti-HAV antibody

1) Plasma collected in 1976 during an outbreak of type A hepatitis at Ft. Richardson, Alaska was used both to coat the microtiter plates and as a source of ^{125}I-labelled antibody. These plasma units were described in the FY 77 annual report.

2) Immune plasma was also obtained from 2 chimpanzees infected with hepatitis A (see Section I.B below).

3) An attempt was made to recover some of the HAV-immune plasma collected under U.S. Army contract during the Joliet prison studies conducted almost a decade ago (Boggs et al., 1970). Publications and records of the Joliet study were reviewed, and a schematic diagram of the study was constructed (Figure 1). A roster of material held by Dr. J.D. Boggs,

Children's Memorial Hospital, Chicago, Illinois, was reviewed, and certain units of plasma identified. Dr. Boggs was visited in Chicago, and has promised to provide most of these plasma units. To date, however, none has been received.

4) Small quantities of paired pre- and post-infection homologous sera, previously collected under US Army contract, were obtained from Dr. S. Krugman of New York University. Additionally, paired sera became available with the successful infection of two chimpanzees (see below). NIH reference reagents V811-801-573 (preinfection chimp) and V811-501-573 (post-infection chimp) were also used.

5) Whole plasma diluted in carbonate buffer pH 9.0 was used to coat the microtiter plates as described below. For radioiodination, IgG was purified by ammonium sulfate precipitation followed by anion exchange chromatography with Sephadex A25. Iodination was carried out by the chloramine-T method, and ^{125}I-antibody was stored at 4°C in 25% fetal bovine serum. Optimum combinations of coat antibody and ^{125}-I antibody were determined by cross comparisons as shown in Table 1.

b. HAV antigen

1) Stool collected from one individual (FJ-1-2) during the Joliet study and shown by other investigators to contain HAV (Mathiesen et al., 1978) was initially used as a source of antigen. Stools (Figure 1) collected on day 33 (FJ-1-2-5) and on day 35 (FJ-1-2-19) after infection are HAV-positive, and have been proven infectious for chimpanzees (see below), while stool collected prior to infection (FJ-1-2-8) served as an antigen-negative control.

2) Antigen was also prepared from chimpanzee stool following the successful infection of two chimpanzees as described below.

3) A crude stool extract was prepared as follows. Stool was diluted 20% w/v in PBS with 0.1% sodium azide pH 7.4, and shaken vigorously with glass beads. Very hard stools were allowed to soften overnight at 4°. Following a low speed centrifugation, supernatant fluid was decanted and the pellet re-extracted with an equal volume of PBS. The supernatant fluids from the two extractions were pooled. This 10% fecal extract was clarified by high speed centrifugation.

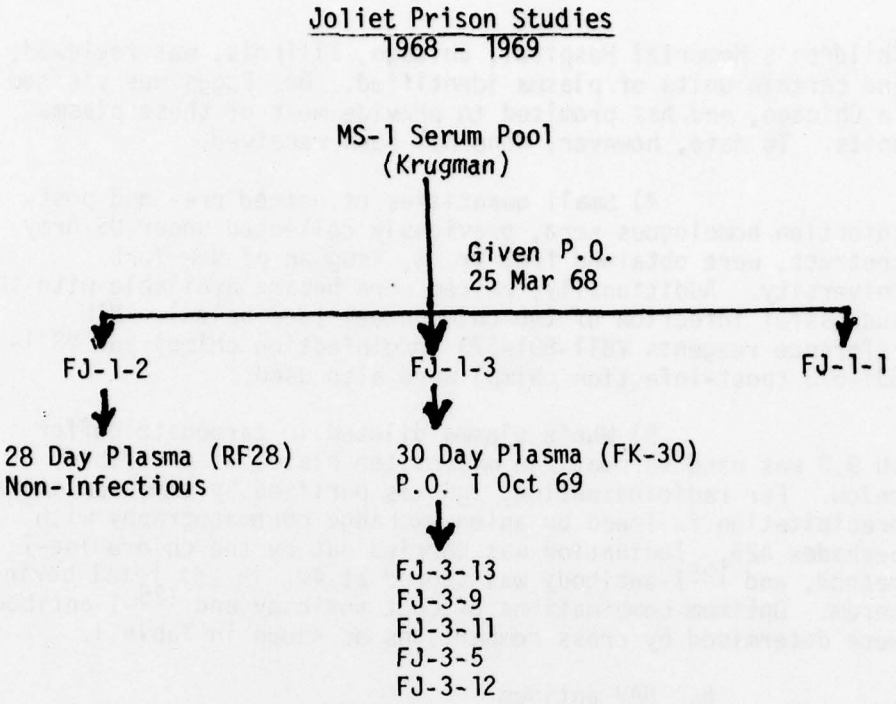


FIGURE 1. Schematic diagram of human infection studies with HAV derived from MS-1 strain of Dr. Krugman. An infectious serum pool was given orally to ten volunteers, three developed clinical hepatitis. Plasma collected 28 days after infection of one of these volunteers (FJ-1-2) did not infect any of ten additional volunteer recipients, whereas 30-day plasma from a second volunteer (FJ-1-3) infected 5 of 10 recipients. Serial stool specimens from all 8 cases of hepatitis are currently stored at WRAIR; stool used to infect the chimpanzees described in section B of this report came from patient FJ-1-2. Stool FJ-1-2-5 was collected 33 days after infection, and stool FJ-1-2-19 was collected 35 days after infection. Plasma is held by Dr. J.D. Boggs, Chicago.

(12000 X G for 30 min), and stored at -70° until use.

3. Radioimmunoassay procedure

The final procedure adopted is outlined in Table 2.

a. Antibody coat and ^{125}I antibody selection

Based on the P/N results expressed in Table 1, the homologous combination AK003 (coat antibody)/AK003 (^{125}I-antibody) was chosen for determination of anti-HAV antibody, while the heterologous (and therefore possibly more specific) AK005/AK003 combination was chosen for antigen detection.

b. Test controls and test interpretation

A combination of controls are included on each SPRIA microtiter plate to assess the specificity of the reaction and the degree of sensitivity of antibody detection. Plates set up to test for antibody include the following controls:

1) Both negative and positive antigen preparations are tested on each plate in the absence of any blocking antisera. A P/N ratio of 2.1 or greater indicates a positive reaction.

2) To assess specificity of the reaction, positive stool is blocked with a panel of four anti-HAV negative sera (diluted 10^{-2} in PBS-pH 7.4) as well as anti-HAV positive sera. A 50% reduction in radioactivity bound in wells blocked with positive sera compared with wells blocked with negative sera indicates a specific reaction.

3) Positive stool is also blocked with a dilution series of anti-HAV positive serum (diluted 10^{-2} to 10^{-4} and adjusted to a constant serum concentration of 10^{-2} with pooled anti-HAV negative serum). To retain comparability between wells it is essential to maintain the total blocking antisera concentration at 10^{-2} as a certain degree of non-specific protein blocking occurs. The greatest dilution of positive serum which still results in a 50% reduction in radioactivity bound indicates the sensitivity of the test in the detection of antibody.

Results from a typical SPRIA plate are shown in Table 3. For plates designed for antigen detection, a modified set of

Table 1. P/N* Ratio Obtained Using Different Combinations of Sera for "Coat Antibody" and "^{125}I-Antibody"

	Coat Antibody					
	AK005	AK019	AK003	AK059	CH267	CH173
AK005	3.7	2.8	3.8	4.1	2.9	1.8
AK019	2.2	2.6	3.6	3.5	2.6	3.3
AK003	5.0	3.2	4.8	4.0	3.0	3.4
AK059	4.3	2.4	3.4	2.5	1.8	3.4

*P/N ratio represents cpm bound by wells containing antigen-positive stool divided by cpm bound by wells containing antigen-negative stool. Six different plasmas were used as coating antibody in combination with 4 different radioiodinated antibody preparations.

Table 2. Procedure for Radioimmunoassay for Hepatitis A Virus

1. Wash flexible microtiter "U" plates 5x in running water, tap dry.

2. Deliver 100λ of immume serum diluted 10^{-2} in pH 9.0 carbonate buffer to each well, and let sit 4 hours at room temperature. Wash plate 3x with PBS-tween 20 0.5%. Allow 5 minutes between washes.

3. Fill wells with bovine serum albumin 1% in PBS pH 7.4, and place at 4°C overnight. Wash 3x with PBS-tween 20.

4. Deliver 20λ of antigen (5-10% clarified stool extract) and place at 4°C for 18-24 hours. Wash 3x with PBS-tween 20.

5. Within 15 minutes of PBS-tween 20 wash, deliver 20λ of blocking (test) antisera, diluted 10^{-2} in PBS pH 7.4, to each well. Let sit 15 minutes at room temperature.

6. Add 20λ of ^{125}I-Antibody (~200,000 cpm/20λ) to each well. Leave at room temperature 2 hours, then wash 5x with PBS-tween 20. Cut out wells and count in a gamma counter.

Table 3: Radioimmunoassay Results: Anti-HAV Detection, Plate A126

Wells	Antigen	Blocking Antiserum	Mean CPM	% Block
B2,3	FJ-1-2-8 (Neg)	None	528	
B4,5	FJ-1-2-19 (Pos)	None	4156[1]	
B6,7	"	GD Pre-Infection (Anti-HAV Neg)	2334	
B8,9	"	FB114 "	2408	
B10,11	"	FB273 "	1983	
C2,3	"	FB310 "	2272	
C4,5	"	GD Post-Infection 10^{-2} (Anti-HAV Pos)	123	94%[2]
C6,7	"	" $10^{-2.5}$ "	251	89%
C8,9	"	" 10^{-3} "	662	71%
C10,11	"	" $10^{-3.5}$ "	1173	48%

[1]The P/N ratio is 7.9.

[2]The "% block" expresses the percent reduction in CPM compared with the mean CPM found in wells blocked with anti-HAV-negative serum, on this plate 2249 CPM. A greater than 50% block indicates an anti-HAV-positive serum.

controls is included. Negative and positive stools are compared as above in the absence of blocking antisera, and a known positive stool is tested when blocked by homologous pre- and post-infection chimpanzee plasma specimens. Stool specimens being tested for antigen are blocked by the same homologous plasma pair, and results are interpreted as above. Results from a typical plate designed for antigen detection are shown in Table 4.

1.25% stool extracts still possessed detectable antigen (Figure 2), but generally 5% to 10% stool extracts generated a higher P/N ratio and greater absolute radioactivity bound. Antigen preparations could be recovered from the plate and used again at a later date, but only with a significant reduction in activity (Figure 3).

After antigen is washed off the plate, the blocking antibody must be delivered to each well within 15 minutes (Table 2,, Step 5). The timing of this step was critical, as longer time intervals were found to significantly decrease the P/N ratio (Figure 4). This may be a manifestation of low afinity of the antibody in the Alaska plasmas AK003 and AK005 for the Joliet antigen.

4. SPRIA Results

a. Detection of anti-HAV

1) Results obtained with paired homologous sera collected pre- and post-infection are shown in Table 5. There was a greater than 50% reduction of cpm by each of the post-infection sera when compared with the homologous pre-infection specimens.

2) Thirty-eight sera, collected during the Ft. Richardson, Alaska outbreak were previously tested by Blaine Hollinger of Baylor University by radioimmunoassay for anti-HAV (see WRAIR annual report FY 77). These sera were retested for anti-HAV using the new radioimmunoassay. The results appear in Figure 5, and show close agreement between the two assays. Several sera, however, which were weakly positive by Dr. Hollinger's assay, were negative when tested at WRAIR. Fewer ambiguous (midrange) results were obtained with the WRAIR assay, and serious disagreement was limited to only one serum, EX 273. This serum sample, a late specimen from an initially anti-HAV negative individual

Table 4. Radioimmunoassay Results: HAV Detection, Plate A117

Well	Antigen	Blocking Anti-Serum	Mean CPM	% Block
B4,5	FJ-1-2-8 (Neg)	None	807	
B6,7	FJ-1-2-19 (Pos)	None	4798[1]	
B8,9	"	CH173 2/9 (Anti-HAV Neg)	3666	
B10,11	"	CH73 3/16 (Anti-HAV Pos)	415	88%[2]

[1] P/N ratio equals 5.9.

[2] % block represents the reduction in CPM between wells containing the same antigen, but blocked with pre-infection (2/9) and post-infection (3/16) chimpanzee serum.

Table 5. Results of Anti-HAV Testing* of Post-HAV Infection Serum Compared with Hemologous Pre-Infection Serum.

Source of Paired Sera	% Block
NIH Reference Chimpanzee	76%
Chimpanzee CH 267	69%
Chimpanzee CH 173	78%
GD (Human)	84%

*Antigen employed was 10% FJ-1-2-5. Paired sera were tested concurrently, and the results expressed represent the reduction in cpm from the pre-infection to the post-infection sample.

Table 6. Anti-HAV Results with Hyland ISG Lots.

Dilution	Control Serum Anti-HAV Positive GD Post	Hyland γ-Globulin 0632D003AA	Hyland γ-Globulin 0632E003AA
1:100	52%	63%	78%
1:300	67%	70%	57%
1:1000	56%	66%	53%
1:3000	0%	43%	60%
1:10,000		39%	20%
1:30,000		28%	49%
1:100,000		6%	31%

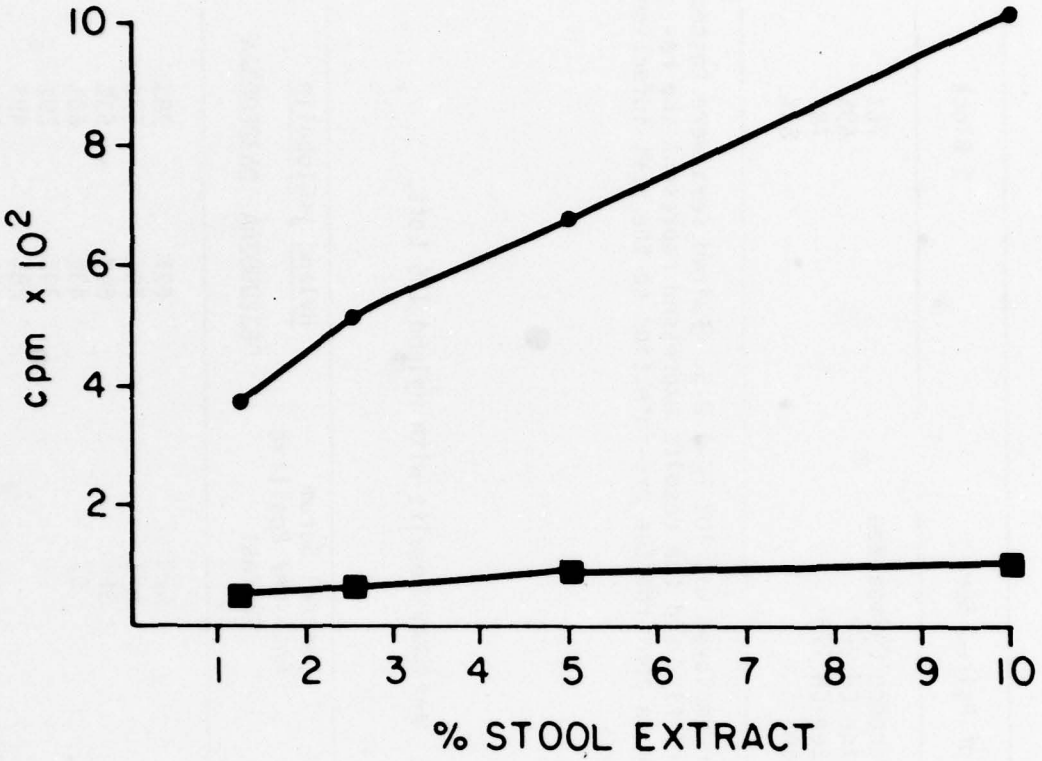


FIGURE 2. Chimpanzee 173 stool extracts tested by SPRIA for HAV in the absence of any blocking antisera. ■—■ pre-infection 2/10/78 stool specimen; ●—● acute phase 3/6/78 stool.

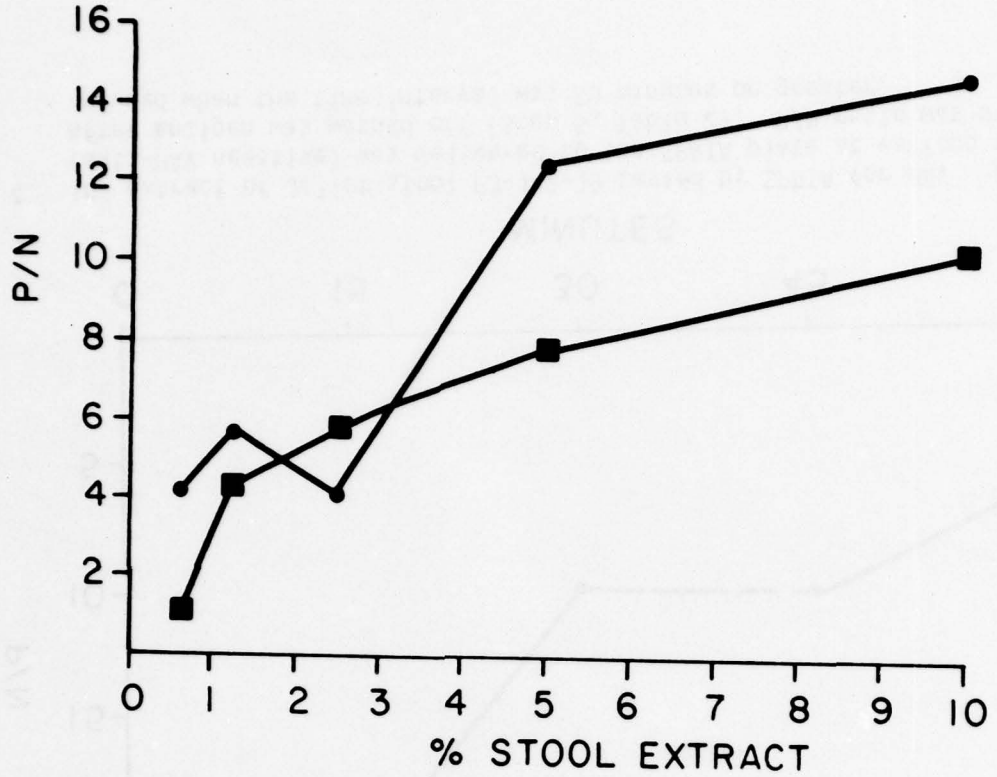


FIGURE 3. Joliet stool FJ-1-2-19 extracts tested by SPRIA for HAV. ●—● stool extract stored at -70° since preparation; ■—■ identical stool preparation previously tested at 10% concentration, recovered from SPRIA plate, frozen at -20°, and retested at various dilutions. Although the recovered antigen is satisfactory at 5-10% concentrations, there is a significant reduction in detectable HAV.

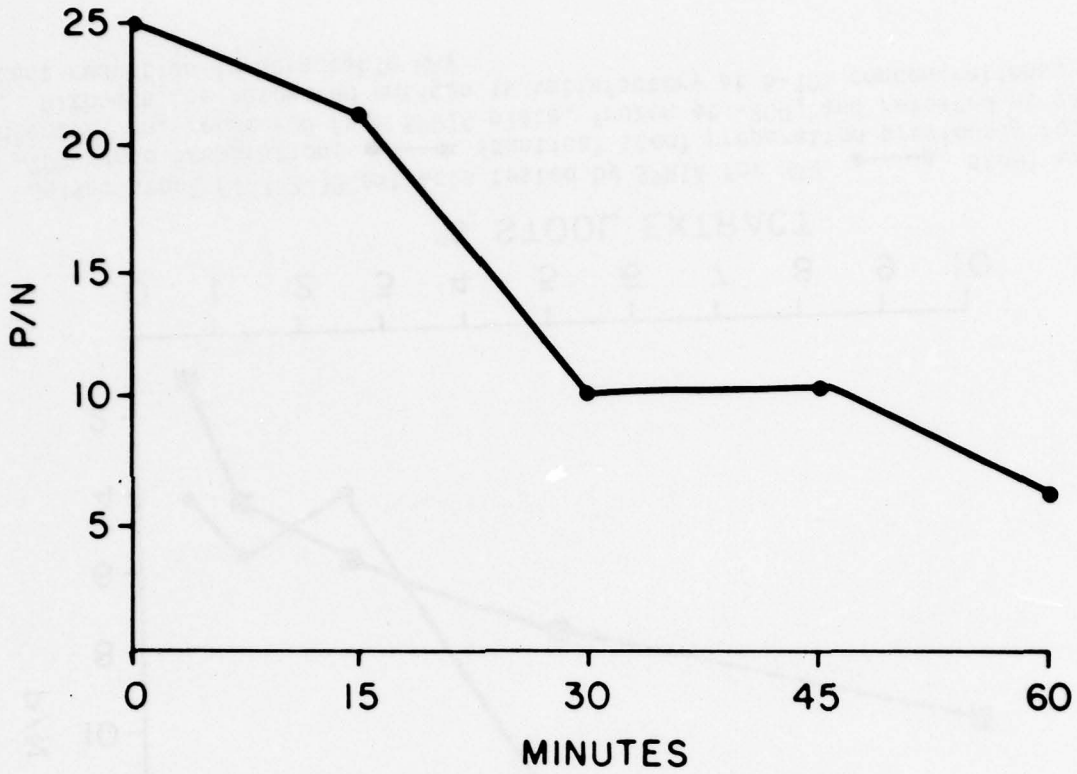


FIGURE 4. 10% extract of Joliet stool FJ-1-2-19 tested by SPRIA for HAV. Blocking serum (anti-HAV negative) was delivered to the SPRIA plate at various time intervals after antigen was washed off (Step 5, Table 2). P/N ratio was significantly reduced when the time interval was 30 minutes or greater.

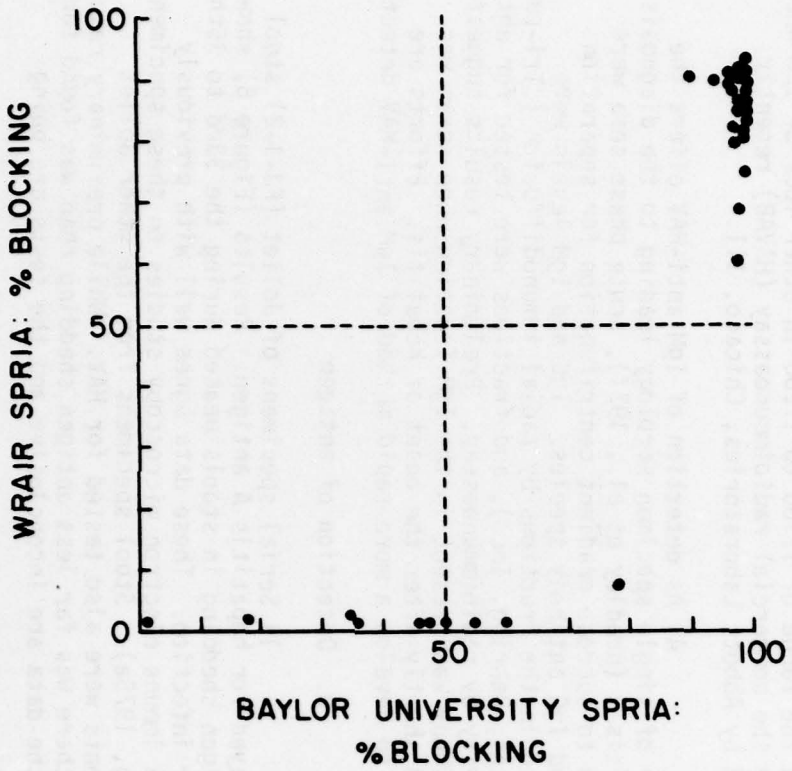


FIGURE 5. Comparison of SPRIA for anti-HAV as performed at WRAIR and at Baylor University. Results are expressed as % inhibition; >50% is considered positive for anti-HAV in both assays. Thirty-eight sera were tested by both methods.

who did not develop clinical evidence of hepatitis, was positive by Hollinger's method (78%) but negative at WRAIR (7%). Further comparison between the two assays is scheduled for the near future.

3) Two lots of immune serum globulin (ISG) currently being used for prevention of hepatitis by the Army were tested for anti-HAV content. Antibody titers, shown in Table 6, were 1:1000 and 1:3000. The Bureau of Biologics has described antibody titers in the range of 1:100 to 1:200 in other lots of ISG when tested by the commercial radioimmunoassay (HAVAB) recently developed by Abbott Laboratories, Chicago, Ill.

4) As detection of IgM anti-HAV offers the advantage of single specimen serology leading to the diagnosis of hepatitis A (Bradley et al., 1977), acute phase sera were subjected to sucrose gradient centrifugation for separation of IgM and IgG antibody species. IgG and IgM levels were determined in the fractions by radial immunodiffusion ("Tri-partigen" plates, Behring, Inc.), and fractions were tested for anti-HAV activity by radioimmunoassay. Preliminary results suggested that antibody was present in the IgM fraction when serum was collected shortly after the onset of hepatitis. Efforts are underway to develop a more rapid method of IgM anti-HAV detection.

b. Detection of antigen

1) Serial specimens of Joliet (FJ-1-2) stool were assayed for hepatitis A antigen. Results (Figure 6) show that antigen shedding in stools peaked during the 33rd to 35th day after infection. These data agree well with previously published immune electron microscopy studies on these specimens (Dienstag, 1975a). Stool specimens from the other Joliet participants were also tested for HAV. While preliminary results suggest there was far less antigen shedding than was found for FJ-1-2, the data are inconclusive and the tests are being repeated.

2) Serial chimpanzee stool specimens were also tested. Results are described in the following section.

B. Infection of Chimpanzees with hepatitis A virus

Two chimpanzees (CH167 and CH263), held at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP),

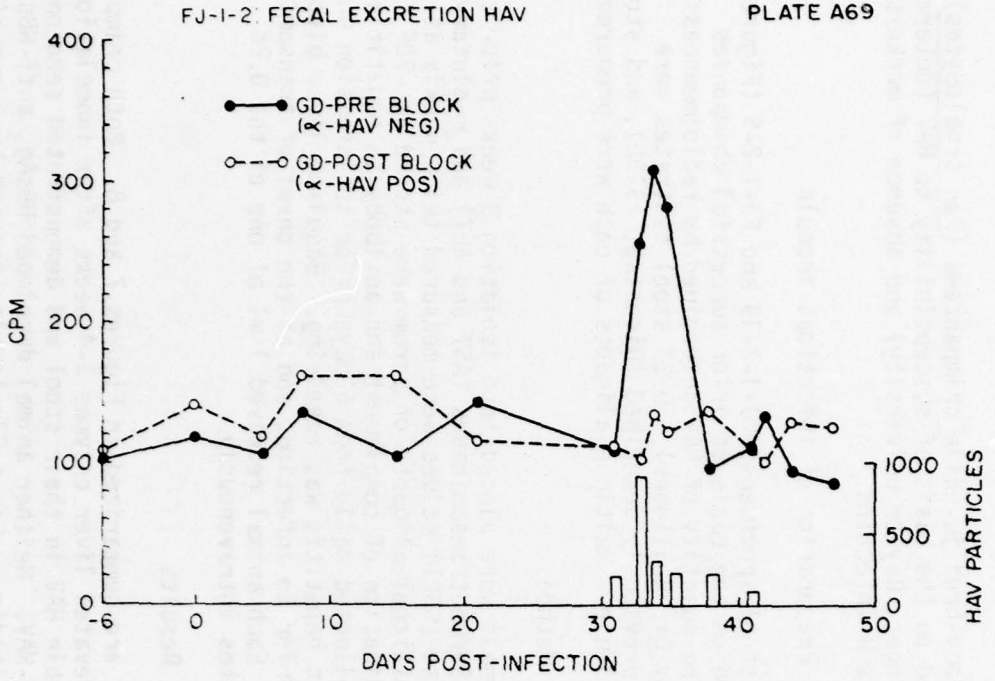


FIGURE 6. Detection of HAV in stool of Joliet volunteer FJ-1-2. 10% stool extracts were tested by SPRIA. Shown for comparison are results obtained by others using immune electron microscopy (IEM) on the same specimens (Dienstag et al., 1975a). ●——● SPRIA results when antigen blocked with HAV-negative serum; O----O results when blocked with HAV positive serum. IEM results (bar graph) are expressed as HAV particles found in 5 squares of a 400 mesh grid.

New York University, were infected with HAV from Joliet stool. Stool specimens were collected from each animal, and provided an additional source of HAV antigen.

1. Selection of animals

Two colony-bred juvenile chimpanzees (Pan troglodytes) were selected on the basis of susceptibility to HAV (determined by Dr. Hollinger, Baylor University) and absence of markers for hepatitis B infection.

2. Preparation of infectious inocula

Joliet stool specimens, FJ-1-2-19 and FJ-1-2-5 (Figure 1), were selected on the basis of prior successful chimpanzee infections and quantity of HAV determined by radioimmunoassay (performed by Dr. Hollinger). 0.2% stool filtrates were prepared as previously described (Dienstag, 1975b), and stored at -70° until use. Multiple aliquots of each were prepared.

3. Methods

The animals were placed into isolation 3 weeks prior to infection. Serum transaminases (AST and ALT) and γ-glutamyl transpeptidase (GGPT) values were measured twice weekly at LEMSIP. Additional aliquots of serum were stored at -70° for later determination of complement and antibody to hepatitis A. Stool was collected daily from 5 days prior to infection until evidence that hepatitis was resolving. Baseline liver biopsies were taken prior to infection, and at the onset of transaminase elevations. Each animal received 1 ml of one of the 0.2% stool filtrates intravenously.

4. Results

Results are summarized in Figures 7 and 8. Both chimpanzees developed elevated liver enzymes 3-4 weeks after inoculation, shed detectable HAV in their stool and demonstrated seroconversion to anti-HAV. Neither animal developed HBsAg, anti-HBs or anti-HBc antibodies. Serum C3c levels (assayed by commercial radial immunodiffusion, Behring, Inc.), fell in both animals coincident with the rise in liver enzymes and the appearance of anti-HAV. This apparent serum complement consumption needs to be confirmed, but suggests a role for immune injury in the production of type A hepatitis.

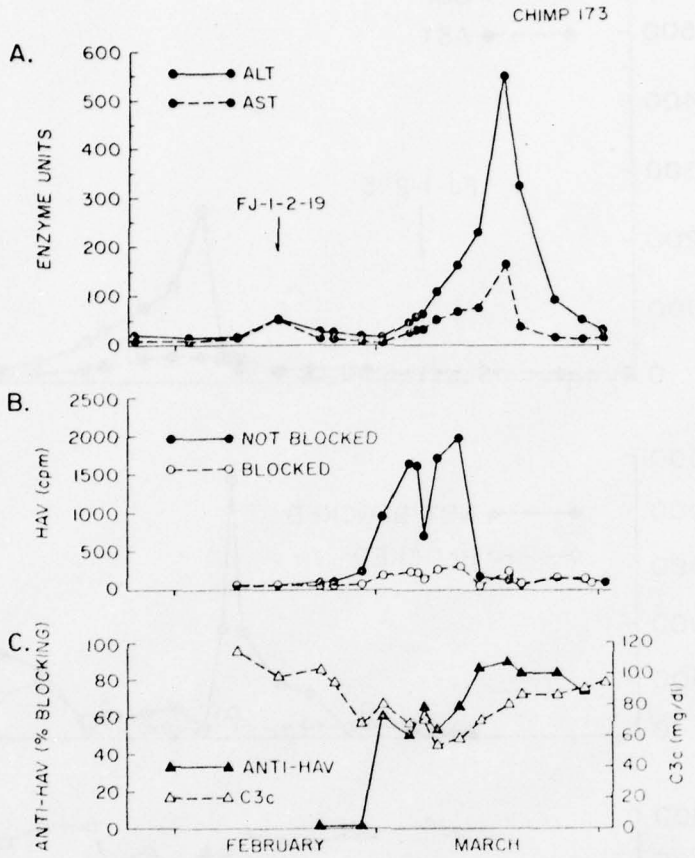


FIGURE 7. Experimental infection of chimpanzee 173 with hepatitis A virus. A) serum AST and ALT values; the animal received 1 ml 0.2% FJ-1-2-19 IV at time indicated. B) fecal shedding of HAV as determined by SPRIA; stool extracts (10%) were blocked with anti-HAV-negative (●——●) and anti-HAV-positive sera (○-----○) to determine the specificity of the reaction. C) anti-HAV (▲——▲) determined by SPRIA, and C3c (△- - -△) measured by commercial radial immunodiffusion.

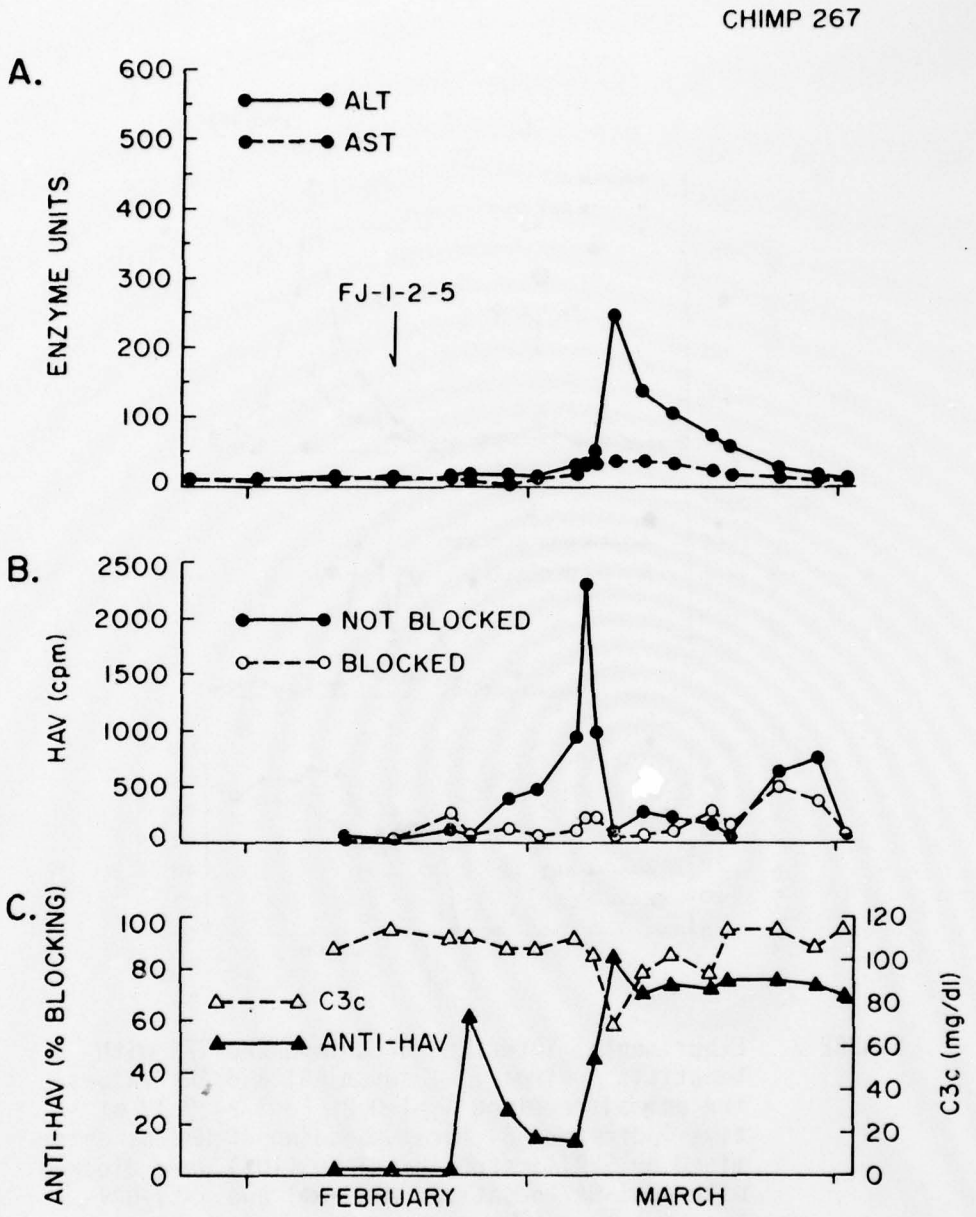


FIGURE 8. Experimental infection of chimpanzee 267 with hepatitis A virus from Joliet stool FJ-1-2-5. See legend to Figure 7.

5. Chimpanzee HAV antigen

CH173 shed HAV in stool for a longer period of time than did CH267 (Figures 7 and 8). Stool collected on day 19 from CH173 was prepared for use in the radioimmunoassay as described in section A2 b3 above, yielding 200 ml of 10% stool extract. This material contains detectable HAV (Figure 2) and, if used at a 10% final concentration, will permit 3000 individual serum antibody determinations. A pre-infection chimpanzee stool lot was also prepared to serve as an HAV-negative antigen control in future assays.

C. HAV purification

Purified hepatitis A virus will be necessary for the development of a radioimmunoassay specific for IgM anti-HAV. Preliminary attempts have included separation by differential centrifugation and CsCl equilibrium gradient centrifugation.

A 10% suspension of Joliet FJ-1-2-5 stool was shaken with glass beads and clarified at 2600 rpm in the PR-6 centrifuge for 10 minutes followed by two additional clarification steps at 8000 rpm in the Sorvall SS-34 rotor for 30 minutes. The supernatant fluid was layered onto a CsCl cushion (σ=1.85 gm/cm^2) and centrifuged for 3 hours at 27,000 rpm in the SW 27.1 rotor. The interface material was collected, dialyzed for 5 hours against PBS pH 7.4 with 1% SDS, and placed over a pre-formed linear CsCl gradient (σ=1.13 -1.52 gms/cm^3). This gradient was centrifuged at 25,000 rpm in the SW 27.1 rotor for 20 hours and 1 ml fractions were collected from the bottom. Fractions were then tested by radioimmunoassay for HAV (Figure 9).

A broad band of HAV activity was found in fractions 7-9 (density 1.27 - 1.33), correlating with the presence of a visible band in the gradient. This material will be further purified by molecular exclusion chromatography (Sepharose 2B) and rate zonal centrifugation.

D. Hepatitis A Outbreak at Schofield Barracks, Hawaii.

During June 1978, an outbreak of hepatitis among soldiers of the 25th Infantry Division was investigated by a team from WRAIR. The results of the epidemiologic investigation are found elsewhere in this report (see Work Unit 001, Epidemiologic Studies of Military Diseases). Twenty-five persons were identified

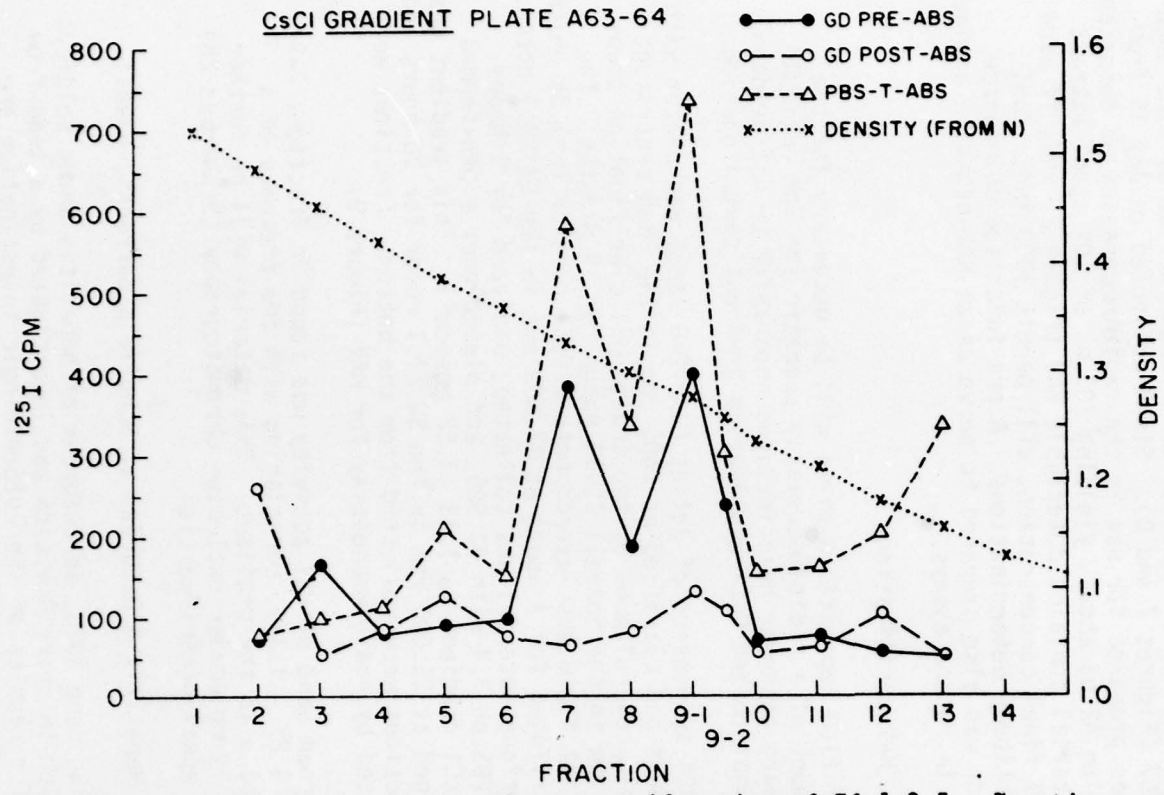


FIGURE 9. Cesium chloride density gradient centrifugation of FJ-1-2-5. Fractions were tested for HAV by SPRIA. Each fraction was tested when blocked by anti-HAV-negative (●——●); and anti-HAV-positive (○- - -○) sera; and PBS-tween 20 (Δ- - -Δ). A broad band of HAV activity is found in the density range of 1.27 to 1.33.

as meeting clinical criteria for hepatitis. Serum was collected from 23 of these cases and from 30 age- and unit-matched asymptomatic controls. These sera were subsequently tested for anti-HAV, HBsAg (AUSRIA II), anti-HBs (Ausab) and anti-HBc by radioimmunoassay. Results are shown in Table 7. Ninety-six percent of the symptomatic individuals were anti-HAV positive, whereas only 50% of the asymptomatic controls had anti-HAV (Figure 10). The results provided serologic confirmation that the outbreak was due to type A hepatitis. The 50% prevalence of anti-HAV found in unaffected personnel is consistent with previously reported studies of similar healthy American populations. Serologic markers for hepatitis B infection were also more common in cases (17%) than controls (7%) suggesting that, in at least a few cases, hepatitis type B may have been the etiologic agent. Anti-HAV positive sera from cases and controls are now being tested for IgM antibody to HAV.

II. Hepatitis B Virus (HBV)

Principal projects have included modification of the radioimmunoassay for HBV core antigen (HBcAg) to improve test specificity, and studies of HBV surface antigen (HBsAg) production by the PLC/PRF/5 cell line in vitro.

A. Modification of the Anti-HBc radioimmunoassay.

1. Purification of HBc from chimpanzee liver.

Chimpanzee 683 (described in previous annual reports) served as a source of HBV-infected liver. HBcAg was purified according to the method of Fields et al. (1976) by homogenization of liver, followed by ether extraction, differential centrifugation, treatment with detergent, and sucrose gradient centrifugation. Fractions of the sucrose gradient were diluted 30-fold and tested for HBsAg (AUSRIA II) and HBsAg by a radioimmunoassay similar to that described in the preceding section for HAV. Results appear in Figure 11. Peak HBcAg activity was found in fraction 15, and was detectable by radioimmunoassay at a dilution of 1:100 (P/N ratio=2.91) and by complement fixation at a final dilution of 1:8. Fractions 14 and 15 of the gradient were pooled, diluted 1:20 and divided into aliquots for use in future assays.

Table 7. Results of Hepatitis Serology: Schofield Barracks Outbreak[†]

Study Group	Number Tested	Anti-HAV	HBsAg	Anti-HBs	Anti-HBc
Hepatitis Cases	23	96%	4%	9%	17%
Unit-matched controls	30	50%	0	7%	7%

[†]Results are expressed as the percentage positive for antibody (or antigen) in each test.

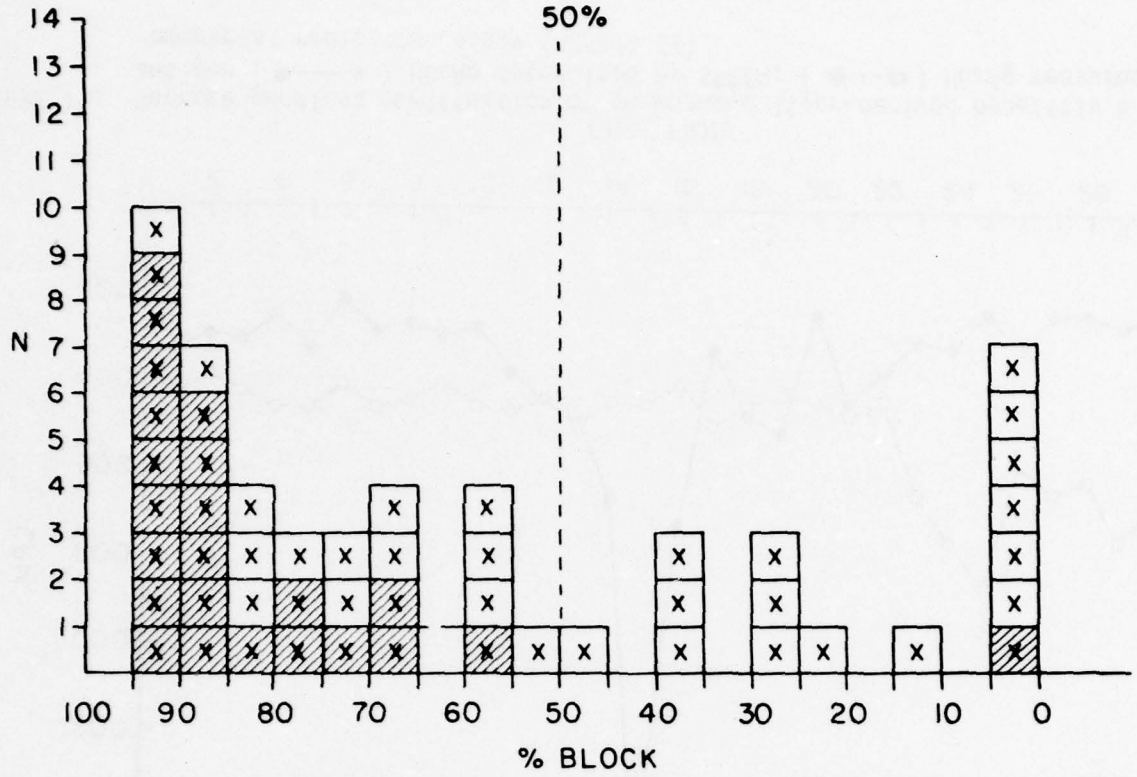


FIGURE 10. Results of anti-HAV testing by SPRIA on sera collected during Schofield Barracks hepatitis outbreak. Each block represents serum taken from one individual. Shaded blocks represent identified cases of hepatitis, whereas unshaded blocks represent asymptomatic matched controls. Greater than 50% blocking activity is indicative of anti-HAV.

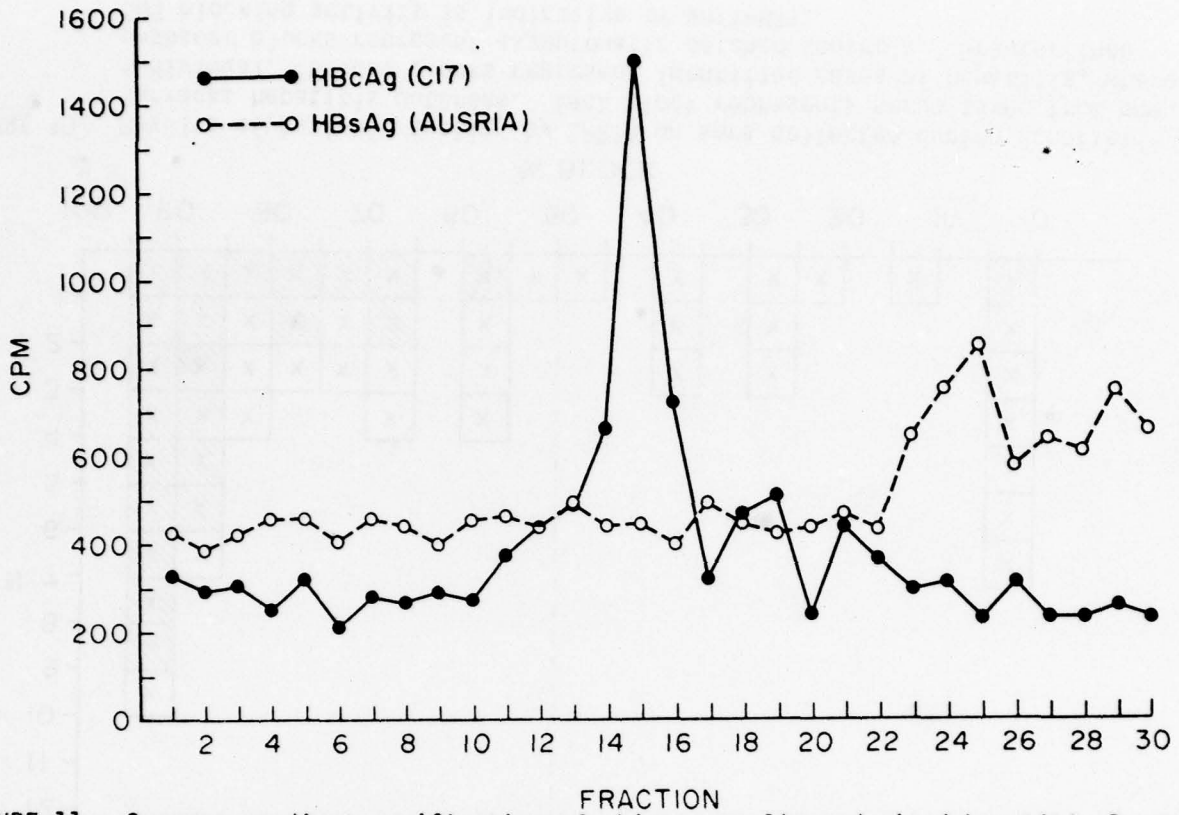


FIGURE 11. Sucrose gradient purification of chimpanzee liver-derived hepatitis B core antigen (●——●) HBcAg determined by SPRIA; (●---○) HBsAg measured by commercial radioimmunoassay (AUSRIA II).

2. SPRIA for anti-HBc

The modified radioimmunoassay for anti-HBc was similar to the HAV assay described above with the exception that normal saline was used in place of PBS-tween. A 50% reduction in bound radioactivity was considered indicative of a positive result for anti-HBc antibody in the blocking serum sample.

3. Results

All sera tested to date which have contained HBsAg (AUSRIA II) have also been positive for anti-HBc. Sera which are positive for anti-HBs (Ausab) may or may not contain anti-HBc activity. Sera collected during the Schofield Barracks outbreak from symptomatic cases and asymptomatic matched controls (see above) were tested for anti-HBc; results are summarized in Table 7.

B. PLC/PRF/5 Hepatoma Cell Line

A continuous cell line (designated PLC/PRF/5), established from the liver of an HBsAg-positive patient with primary liver carcinoma, was provided by Dr. Jenifer Alexander of South Africa (Macnab, 1976). This material was received at the 63rd passage level and subjected to the following studies.

1. Confirmation of HBsAg production in vitro.

Cells were grown in Eagles MEM with Earle's salts, supplemented with 5% fetal calf serum, glutamine, pyruvate and non-essential amino acids. At intervals, media was removed, clarified at 200 rpm x 10 minutes in the PR-6 centrifuge, and tested for HBsAg by commercial radioimmunoassay (AUSRIA II). HBsAg was continuously released into the culture media as shown in Figure 12. HBsAg antigen was detectable at a dilution of 10^{-2}, whereas similar media taken from Vero cell cultures contained no detectable antigen. HBsAg reactivity in the AUSRIA II test was totally abolished by absorbtion with rabbit antisera previously prepared to highly purified HBsAg (subtype adw), but not with normal rabbit serum. HBcAg could not be detected in media by radioimmunoassay, nor in the cells themselves using an indirect fluorescent antibody technique.

Media from PLC/PRF/5 cultures were clarified at 8000 x g for 30 minutes, layered onto a 32 ml linear CsCl gradient (σ1.15-1.45 gms/cm^3) and centrifuged in an SW25 rotor at 24,000

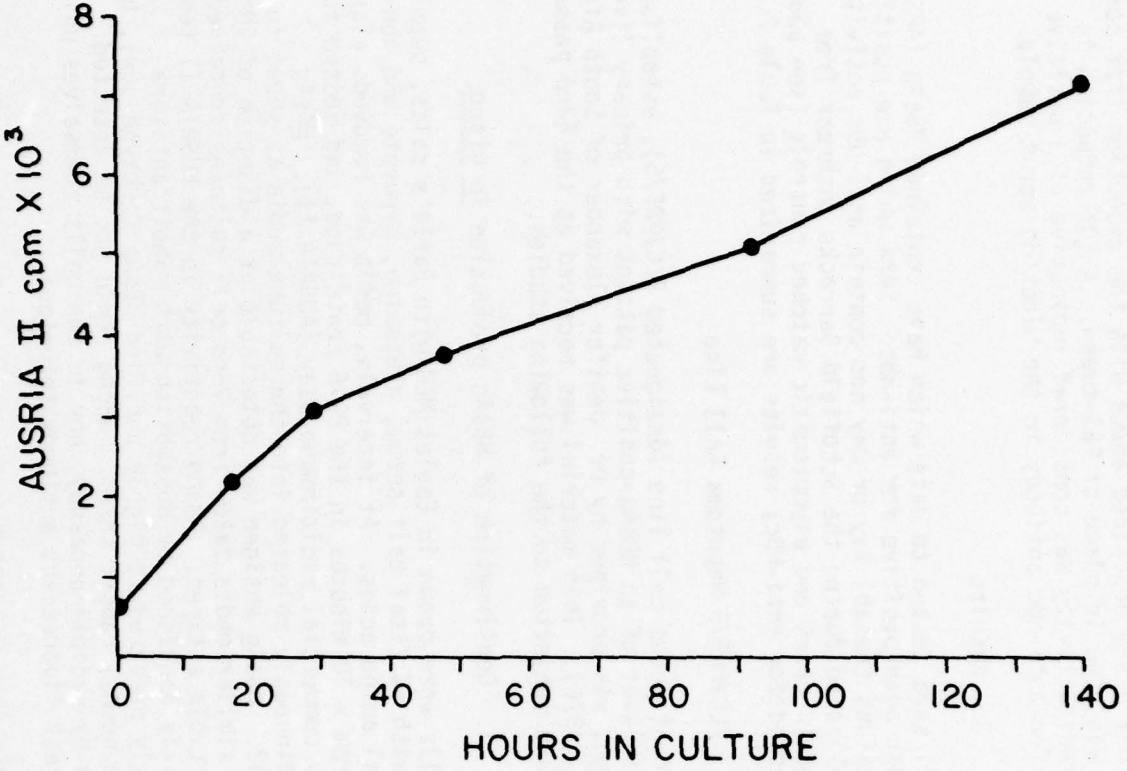


FIGURE 12. Release of HBsAg into culture media by PLC/PRF/5 cells. HBsAg was measured by commercial radioimmunoassay (AUSRIA II).

rpm for 22 hours. HBsAg was found at a density of 1.18-1.22 gm/cm^3, close to the reported bouyant density of plasma-derived HBsAg. These studies confirm that this cell line produces HBsAg which is immunologically and physically indistinguishable from HBsAg recovered directly from infected persons.

2. Effect of Ribavirin (Virazole) on HBsAg production.

Because ribavirin has been reported to have a beneficial effect on both acute and chronic hepatitis B infections, the effect of this drug on HBsAg production by this cell line was investigated. Cells were grown to 80% confluence in 24 well Linbro plates and were fed with media containing various concentrations of ribavirin and d-actinomycin. Because cells continued to release HBsAg for up to 24 hours in the presence of optimal concentrations of d-actinomycin, the drug-containing media was replaced after 24 hours. Culture media was then collected at 24-hour intervals and tested for HBsAg.

As shown in Figure 13, ribavirin treatment resulted in an incomplete, but dose-related inhibition of HBsAg production. Very high levels of ribavirin (200 ugm/ml) resulted in approximately 60% inhibition of HBsAg production. However, identical concentrations of ribavirin resulted in similar inhibition of cellular protein synthesis, as assessed by ^3H-amino acid incorporation into TCA-precipitable material. Cellular RNA synthesis (^3H-uridine) incorporation was reduced to an even greater degree. Thus, the effect of ribavirin on HBsAg production was non-specific and probably due to impaired cellular RNA synthesis.

3. Effect of Adenine Arabinoside (Vidarabrine, Ara-A) on HBsAg production.

Adenine arabinoside has also been reported to have a beneficial effect on chronic hepatitis B infection and therefore the effect of adenine arabinoside on in vitro production of HBsAg was studied using similar methods. Less dose-dependent inhibition of HBsAg production was found with adenine arabinoside than was seen with ribavirin (Figure 13). Adenine arabinoside also caused a quantitatively similar reduction in cellular protein synthesis at these concentrations indicating that, like ribavirin, its effect on HBsAg production was non-specific.

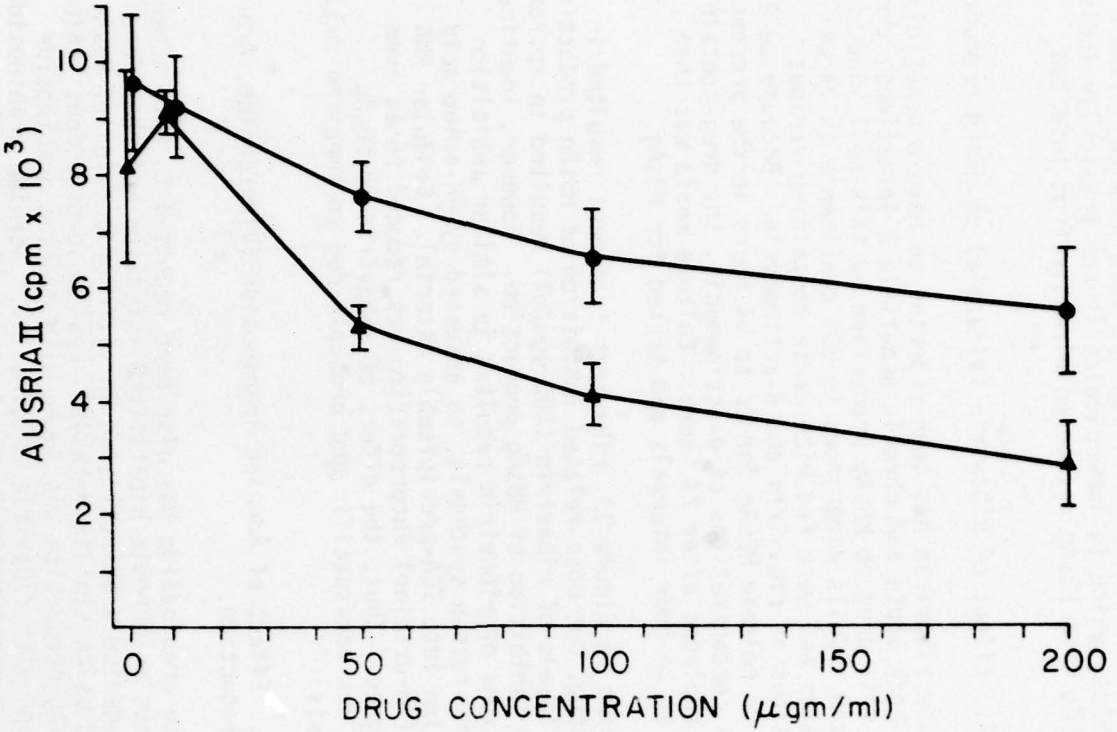


FIGURE 13. Effect of various concentrations of ribavirin (▲——▲) and adenine arabinoside (●——●) on production of HBsAg by PLC/PRF/5 cells in vitro. Cells were pre-treated with each drug for 24 hours, then grown for an additional 4 days in media containing the drugs. Finally, media were collected and tested for HBsAg by AUSRIA II.

4. Effect of human interferon on HBsAg production.

A single experiment was conducted to determine if human interferon (NIH reference reagent) would inhibit HBsAg production. Interferon (100 NIH reference units/ml) effected a 50% reduction in HBsAg production, but inhibited total protein synthesis by at least 20%.

C. Subtyping of HBsAg-positive South Pacific sera

In collaboration with Dr. Arwin Diwan, University of Hawaii, 58 serum samples (all HBsAg-positive by radioimmunoassay) from the Kingdom of Tonga were studied for HBsAg subtype by immunodiffusion. HBsAg-positive sera from American Samoa and the Cook Islands were also studied. The peoples of these islands are being considered for future trials of an HBV vaccine, and preliminary information was required on the prevalent subtypes of hepatitis B virus. Twenty-one of the Tonga sera contained HBsAg detectable by immunodiffusion. Of these, 14 were identified as ad or adr, and 4 were identified as ay. Three could not be typed. Only 5 sera from the Cook Islands and American Samoa contained sufficient HBsAg to detect by immunodiffusion, and all of these were subtype ay.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 135, Mechanisms of Transmission of Hepatitis Viruses

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6448	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	136
b. CONTRIBUTING				
c. XXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Development of Biological Products

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	4	357
	CURRENT 79	4	439

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: Altieri, P.L. DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Bioassays; (U) Biological Products; (U) Dengue Virus Vaccine; (U) Febrile Antigens; (U) Meningococcal Vaccines; (U) Pseudomonas Vaccine; (U) Vaccines.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This work unit is concerned with the development of manufacturing methods and the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

24. (U) Increased effectiveness and reduced reactivity are pursued by use of new physical and chemical methods for processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.

25. (U) 77 10 - 78 09 - Investigations on the development of new and improved biological products for military use have continued. - 1. Studies on production methods for the preparation of a protein-polysaccharide group B meningococcal vaccine have emphasized the reduction in pyrogenicity by alterations in the growth medium. 2. Investigations were initiated on the pilot scale production of a polysaccharide vaccine from Pseudomonas aeroginosa, type 1. 3. The development of a dengue virus type 2 vaccine was continued. In addition, the cloning of dengue virus type 3 was carried out to obtain a candidate virus for vaccine production. 4. An evaluation of the stability of freeze-dried diagnostic antigens dispensed in plastic wells was initiated. 5. Adaptation of a mouse protection test for the evaluation of meningococcal vaccines was commenced. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 136 Development of Biological Products

Investigators.

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Associate: Sanford Berman, Ph.D.; Patricia L. Altieri, B.S.; Doria R. Dubois, M.S.; Calvin J. Powell, Jr., M.S.; SP5 Dolores S. Brooks, B.A.; Melanie G. Butler, B.S.; Clayton R. DeSett, B.S.; Clayton Hartley, B.S.; Quesada Jackson; SP4 Patricia B. Matix, B.S.; Alice Strong, B.S.; Robert L. Timchak; SP4 Cheryl L. Tjiattas, B.A.; Gary A. Vincent, B.S.; Bernard Young.

Description

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress

1. Meningococcal Vaccines

During this period laboratory studies were continued on Neisseria meningitidis groups Y and 135, for the purpose of obtaining appropriate purified polysaccharide preparations for use in the immunization of humans against these types of meningitis. In addition to the monovalent vaccines, a combined vaccine containing the polysaccharides from these two groups was prepared for evaluation in man. Investigations also continued on improved media and processing methods for the production of purified protein-polysaccharide complex antigens derived from group B meningococcus, for use as an immunogen.

a. A production lot of the group Y (strain 80Y, Boshard) of N. meningitidis polysaccharide was prepared for evaluation of immunogenicity in man. The culture was grown for 16 hours in modified Franz medium and was processed as previously described (Annual Report, 1977). Some difficulty had been experienced in sterilizing the final product solution by passage through a sterile 0.2 μm membrane filter. This problem was overcome by dissolving the purified polysaccharide in membrane-filtered water at a concentration

of 0.5 mg/ml and passage through 1.2 μm and 0.45 μm membrane filters before the 0.2 μm sterilizing filter. The product was filled at 2.5 mg polysaccharide per vial and freeze-dried. Results of *in vitro* assays of the final product are shown in Table I.

Table I

Assays on Group Y (80Y, Boshard) Polysaccharide Vaccine, Lot 1

Yield (mg/L culture)	12.0
Protein (%)	0.80
Nucleic Acid (%)	0.92
Sialic Acid (%)	50.0
Carbohydrate (%)	30.6
Molecular Weight	>100,000
Pyrogenicity	Non-Pyrogenic at 2.5 μg/kg

The above vaccine was tested in man (50 μg dose) by the Department of Bacterial Diseases, WRAIR, and was found to be immunogenic.

b. Additional studies of the group 135 (strain 135-111) of *N. meningitidis* failed to provide a satisfactory method for the production of a purified polysaccharide which would be a suitable immunogen for human use. Therefore, new studies were initiated using another group 135 strain (22-W135).

A 16-hour culture of strain 22-W135 was grown in modified Franz medium and inactivated with Cetavlon. The sediment collected by Sharples centrifugation was divided into two parts in order to compare the usual calcium chloride extraction procedure with a sodium chloride extraction of the crude polysaccharide. The results of both extractions are given in Table II.

Table II

Comparison of Crude Polysaccharide Yields Obtained With Sodium Chloride and Calcium Chloride Extraction of Sedimented N. Meningitidis Strain 22-W135

In Vitro Assay	0.5M NaCl Extract	1.0M $CaCl_2 \cdot 2H_2O$ Extract
Yield (mg/L culture)	68	86
Protein (%)	17.7	26.4
Nucleic Acid (%)	4.4	2.9
Sialic Acid (%)	39.7	34.5
Molecular Weight	<100,000	>100,000

Although the molecular weight of the NaCl extract was less than 100,000, it was decided to carry the material through to a final product in order to complete the comparison.

The crude polysaccharides obtained were each dissolved in a 1/10 dilution of saturated sodium acetate (pH 7.0) at a concentration of 10 mg/ml. They were then fractionated three times with equal volumes of a 75% phenol solution, and the phenol layers were washed sequentially with one volume of 1/10 saturated sodium acetate. After dialysis against distilled water at 5°C, the polysaccharides were precipitated with 80% ethanol in the presence of sodium or calcium, respectively. The results of the phenol extractions are given in Table III.

Table III

Effect of 75% Phenol Fractionation of
Crude Polysaccharide Extracts

In Vitro Assays	0.5M NaCl	1.0M $CaCl_2 \cdot 2H_2O$
Yield (mg/L culture)	38	40
Protein (%)	2.7	2.3
Nucleic Acid (%)	3.5	1.5
Sialic Acid (%)	54.1	59.7
Molecular Weight	<50,000	>100,000

Although the protein content of the calcium extract was 8% higher than that of the sodium extract, the phenol extraction brought both preparations to the same point. Here again, the molecular weight of the sodium extract was not satisfactory (less than 100,000 daltons).

The powder from each phenol-fractionated preparation was then dissolved in water at a concentration of 1 mg/ml for centrifugation at 100,000 x g for six hours. (Previous studies have shown that high speed centrifugation at higher concentrations, or in the presence of any salt, or combined with ethanol opalescence, caused a considerable loss of polysaccharide with little reduction in the protein and nucleic acid content. The six hour centrifugation appeared to be optimum for achieving the desired results). A comparison of the two final products is shown in Table IV.

Table IV

Comparison of Purified Polysaccharides
Derived from Sodium and Calcium Extractions

In Vitro Assays	0.5M NaCl	1.0M $CaCl_2 \cdot 2H_2O$
Yield (mg/L culture)	31	34
Protein (%)	0.84	0.98
Nucleic Acid (%)	2.55	1.01
Sialic Acid (%)	41.5	59.5
Molecular Weight	>100,000	>100,000

In the final product, the sodium-extracted polysaccharide had a satisfactory molecular weight, but had a low sialic acid content combined with high nucleic acid. The calcium-extracted polysaccharide appeared satisfactory.

A production lot was then processed as described for the calcium-extracted polysaccharide. However, a second centrifugation at 100,000 x g for six hours was required to achieve the desired levels of protein, nucleic acid and sialic acid. The purified polysaccharide was sterilized by filtration through a 0.2 µm membrane filter, filled at 2.5 mg polysaccharide per vial and freeze-dried. In vitro assays of the final product are shown in the following table.

Table V

Assays on Group 135 (22-W135) Polysaccharide
Vaccine, Lot 1

Yield (mg/Lculture)	22
Protein (%)	0.98
Nucleic Acid (%)	0.88
Sialic Acid (%)	48.7
Carbohydrate (%)	28.2
Molecular Weight	>100,000
Pyrogenicity	Non-Pyrogenic at 0.25 µg/kg

This material has been tested in man (50 μg dose) by the Department of Bacterial Diseases, WRAIR, and found to be immunogenic.

c. In addition to individual polysaccharide vaccines derived from group Y and group 135 strains of N. meningitidis, the Department of Bacterial Diseases, WRAIR, requested a combined group Y and group 135 vaccine for evaluation in man. For this purpose, 125 mg of group Y (strain 80Y, Boshard) polysaccharide and 125 mg of group 135 (strain 22-W135) polysaccharide were dissolved in 500 ml of membrane-filtered water to give a solution containing 0.25 mg of each polysaccharide per ml. This was clarified by membrane filtration, first through a sterile 1.2 μm filter over a 0.45 μm filter (142 mm diameter). Final sterile filtration was accomplished by passage through a sterile 0.45 μm filter over a sterile 0.2 μm filter (293 mm diameter). In vitro assays of the combined product before and after sterile filtration are given in the following table.

Table VI

Assays of Combined Group Y and Group 135
Polysaccharide Vaccine

	Sterile Filtration	
	Before	After
Sialic Acid (μg/ml)	287	82.3
Nucleic Acid (μg/ml)	5.3	2.7
Carbohydrate (μg/ml)	175.0	57.0

In order to compensate for the loss of polysaccharide incurred during filtration, a fill volume of 15.2 ml was required to yield 2.5 mg combined polysaccharide per vial. Assays of the final, freeze-dried product are shown in Table VII

Table VII

Assays of Combined Group Y and Group 135
Vaccine, Lot 1 (Freeze-Dried)

Protein (%)	1.36
Nucleic Acid (%)	1.44
Sialic Acid (%)	48.0
Carbohydrate (%)	28.3
Molecular Weight	>100,000
Pyrogenicity	Non-Pyrogenic at 0.50 µg/kg

It is postulated that when the polysaccharides from these two strains are combined in solution, some of the molecules form an addition product of such a size that they cannot pass through the pores of the 0.2 µm membrane filter, resulting in a considerable loss of polysaccharide in the filtrate. In the future, if combined vaccines are to be prepared, each polysaccharide preparation will be sterilized separately by filtration, and then combined under sterile conditions before filling and freeze-drying.

d. *The investigations on the production of a protein-polysaccharide antigen prepared from the B-11 strain of* N. meningitidis included the study of a growth medium utilizing sodium glycerophosphate in place of the sodium phosphate and dextrose in the modified Franz medium. This medium (CAG) is similar to the modified Franz medium with 3X casamino acids (CA) and 1/5 dextrose (Annual Report, 1977) in that the final culture pH is basic, which is essential to prevent the breakdown of the high molecular weight group B polysaccharide. In addition, this CAG medium is sterilizable by autoclaving and serves as an excellent growth medium for the B-11 strain.

The CAG medium was investigated as a suitable growth medium for the B-11 strain after sterilization by filtration or by steam under pressure. Sixteen hour cultures (15L) were inactivated with Cetavlon and the precipitates were collected by Sharples centrifugation. The Sharples sediment from each 15L was processed in the same manner as previously described for B-11 protein-polysaccharide, Lot PA-3 (Annual Report, 1977). A comparison of the crude protein-polysaccharide complex from each 15L is given in table VIII.

Table VIII

Comparison of Crude Protein-Polysaccharide Yields
in CAG Medium Sterilized by Filtration or Autoclaving

In Vitro Assays	Filtered	Autoclaved
PH	8.3	8.5
Sharples sediment (gm)	40	44
Protein (mg/ml)	1.27	1.25
Nucleic Acid (mg/ml)	0.22	0.24
Sialic Acid (mg/ml)	0.63	0.65
Molecular Weight	>100,000	>100,000
Ratio Protein:Sialic Acid	1:0.50	1:0.52

The yield of the crude protein-polysaccharide complex is essentially the same. In both instances the protein content is double that of the sialic acid.

The crude preparations were then processed to a final stage, sterilized by filtration through 0.3 μm membrane filters and freeze-dried. A comparison of the two final products is shown in the following table.

Table IX

Comparison of Yields of Protein-Polysaccharide Complex
from CAG Medium Sterilized by Filtration or Autoclaving

In Vitro Assays	Filtered	Autoclaved
Yield (mg/L culture)	200	200
Protein (%)	70.1	65.3
Nucleic Acid (%)	0.45	0.41
Sialic Acid (%)	25.0	29.1
Molecular Weight	>100,000	>100,000
Ratio Protein:Sialic Acid	1:0.36	1:0.45
Pyrogenicity	ND	Non-Pyrogenic at 15 μg/kg

The results indicate that there were no significant differences between the protein-polysaccharide complexes processed from the CAG medium sterilized by filtration or autoclaving.

The complex obtained from the autoclaved CAG medium was then compared with that obtained from the modified Franz medium with 3X CA and 1/5 dextrose (Lot PA-3). The comparison is shown below.

Table X

Comparison of Protein-Polysaccharide Complex
Derived from CAG Medium and Lot PA-3

In Vitro Assays	CAG Autoclaved	Lot PA-3
Yield (mg/L culture)	200.0	56.7
Protein (%)	65.3	36.6
Nucleic Acid (%)	0.41	0.09
Sialic Acid (%)	29.1	57.3
Molecular Weight	>100,000	>100,000
Ratio Protein:Sialic Acid	1:0.45	1:1.56
Pyrogenicity	Non-Pyrogenic At	
	15µg/kg	0.375µg/kg
	Pyrogenic At	
	25µg/kg	0.50µg/kg

The protein:sialic acid ratios and the pyrogenicities are considerably different. In addition, more of the protein in the CAG preparation is outer-membrane protein (Type 2) than in Lot PA-3. It is postulated that this high molecular weight protein may be an important constituent of the vaccine, in addition to the high molecular weight sialic acid molecule.

A production lot of the B-11 strain protein-polysaccharide complex, prepared in the CAG growth medium, is currently being processed for evaluation of immunogenicity in man.

e. Another production lot of the B-11 strain of N. meningitidis was prepared in modified Franz medium with 3X CA and 1/5 dextrose. After inactivation with Cetavlon, this culture was processed to a protein-polysaccharide antigen suitable for human use by personnel of the Department of Bacterial Diseases, WRAIR (Lot BP2-4). From the 205 gm of Sharples sediment (30L culture), 25 gm was removed to be processed in the same manner as Lot PA-3 for comparison purposes only. In vitro assays of both preparations at the final product stage are given in Table XI.

Table XI

Comparison of Protein-Polysaccharide Complexes:
B-11 Preparation (1978) and Lot PA-3 (1977)

In Vitro Assays	B-11 (1978)	Lot PA-3 (1977)
Yield (mg/l culture)	70.0	56.7
Protein (%)	46.7	36.6
Nucleic Acid (%)	0.30	0.09
Sialic Acid (%)	47.4	57.3
Molecular Weight	>100,000	>100,000
Ratio Protein:Sialic Acid	1:1.0	1:1.56
Pyrogenicity		Non-Pyrogenic At
	1.5 µg/kg	0.375 µg/kg
		Pyrogenic At
	3.0 µg/kg	0.50 µg/kg

In the 1978 preparation, it can be seen that the protein: sialic acid ratio is 1:1 as compared to 1:1,56 for Lot PA-3. Moreover, this preparation has much less pyrogen than vaccine Lot PA-3, although it is not as pyrogen-free as is the complex derived from the CAG medium described above. This difference in pyrogenicity was also observed with vaccine lot WZ-2 and Lot BP2-4 (non-pyrogenic at 1.0μg/kg versus 5.0μg/kg).

The only observable difference between the 1977 and 1978 production lots was the pH of the 16 hour cultures. The 1978 lot had a pH of 8.0 to 8.3, whereas the 1977 culture had a pH of 7.6 to 7.8 Studies are planned to determine the significance of this difference in culture pH.

f. At the request of personnel of the Department of Bacterial Diseases, WRAIR, this department prepared 14 hour cultures of the B-11 strain of N. meningitidis for processing to a purified protein-polysaccharide antigen using the methods developed by W. Zollinger.

Two 15L cultures were grown in modified Franz medium with 3X CA and 1/5 dextrose. The 14 hour cultures reached a pH of 7.8 and 7.1 and yielded an average Sharples sediment of 67.5 gm/15L following inactivation with Cetavlon. (A 16 hour culture yields 90 to 100 gm/15L). After processing, the protein-polysaccharide complex was chromatographed on a Sephadex G-100 colunm before final precipitation and freeze-drying (Cetavlon protein-polysaccharide).

Another two 15L cultures were grown in the same medium as above and the 14 hour cultures reached pH values of 7.7 and 7.9. These were inactivated with phenol at a final concentration of 0.5% phenol. The 30L of phenol-inactivated suspension were passed through the B-16 continuous-flow rotor of the Spinco L4 centrifuge operating at 73,340 x g, at a flow rate of approximately 9.6L/hr. The yield of pelleted organisms was 404 gm (wet weight). The outer-membrane protein was obtained from this material and was chromatographed on a Sephadex G-100 column before final precipitation and freeze-drying (Protein).

The crude polysaccharide in the supernatant from the B-16 centrifugation was precipitated with Cetavlon. After passage through the Sharples centrifuge, 59 gm of crude polysaccharide were obtained. This sediment was processed in the same manner as the Cetavlon sediment. The polysaccharide was chromatographed on a Sephadex G-100 column before final precipitation and freeze-drying (Phenol polysaccharide).

In vitro assays of the three preparations are shown in the table below.

Table XII

Assays of Preparations from 14-Hour Cultures of B-11 Strain of N. Meningitidis

In Vitro Assays	Cetavlon Protein-Polysaccharide	Phenol Protein	Phenol Polysaccharide
Yield (mg/L culture)	30.1	44.8	20.2
Protein (%)	17.7	84.0	12.4
Sialic Acid (%)	69.7	None Detectable	97.1
Molecular Weight	>100,000	>100,000	>100,000

Since a protein-polysaccharide complex was desired, it was of interest to know whether or not the protein and polysaccharide would complex in a water solution when combined. Accordingly, equal amounts of the protein and the phenol polysaccharide were dissolved in water to give 964 µg protein and 971 µg sialic acid per ml. Six mg of this mixture were placed on a Sepharose 4B column using 0.1 M Tris, pH 8.0 as the buffer, collecting 2 ml samples. No measurable protein (UV spectroscopy) or sialic acid (Svennerholm) were found in the eluate, thus indicating that a complex had formed.

Next, a 5 mg sample of this complex in 2% DOC (2% deoxycolic acid, 0.05 M glycine, 0.005 M EDTA, pH 8.8) was placed on the Sepharose 4B column using the same buffer as the previous run. In this case the 2% DOC caused the complex to dissociate and be chromatographed. Protein and sialic acid assays indicated that the molecular weights of both components were greater than 100,000, with the main peaks appearing in the void volume at the same point .

Pyrogen tests were performed in rabbits using this protein-polysaccharide complex. The material was non-pyrogenic at 7.5 µg/kg, the highest concentration tested to date.

2. Pseudomonas Vaccines.

During the past year, in collaboration with the Department of Bacterial Diseases, a study was initiated on the development of methods for the pilot-scale production of a polysaccharide vaccine

derived from cultures of Pseudomonas aeruginosa, type 1.

Growth of the type 1 strain of P. aeruginosa was harvested from Tryptic Soy agar plates and transferred to 100 ml of Tryptic Soy broth (containing 3% gylcerol) in 500 ml flasks. These were incubated at 36°C on a reciprocal shaker operating at 110-120 cycles/min. After 4 hours, the 100 ml cultures were transferred to 1.4 L of Tryptic Soy broth (containing 3% glycerol and 0.5 ml of UCON antifoam) in 4 L flasks. These were incubated at 36°C with shaking. After 48 hours, the cultures were removed from the incubator, and to one 1500 ml culture, 15 ml of a 10% solution of Cetavlon was added, and to a second culture, 8.4 ml of 90% phenol solution. The inactivating cultures were thoroughly mixed and were held at room temperature. Samples removed after 15, 30, 120 and 180 min, and again after 18 hours, were plated on Tryptic Soy agar plates. After 18 hours incubation, the plates were observed for growth. The results are summarized in the following table.

Table XIII

Inactivation of Cultures of P. aeruginosa (Type 1)

Inactivation Time	Growth After Inactivation With: Cetavlon	Phenol
15 min	4+	4+
30 min	3-4+	3-4+
1 hr	3-4+	1-2+
2 hr	3-4+	1-2+
3 hr	1-2+	1+
18 hr	1+	-*

* No apparent growth

The above results demonstrate the relative ineffectiveness of Cetavlon for inactivating cultures of P. aeruginosa in a reasonable length of time. On the other hand, after 18 hours of inactivation at room temperature with phenol, the cultures show no viable organisms and thus can be safely handled through the purification procedures. These results were confirmed with 15L cultures of the organism.

When large volumes of the cultures were prepared, it was found that additional UCON was required after 24 hours incubation to prevent foaming to the point of interfering with aeration. Also, before the large volumes of culture could be passed through 0.45μm

concentrator plates, it was necessary to remove the organisms by high speed centrifugation, first through a Sharples centrifuge and then through a continuous flow centrifuge operating at 100,000 x g.

Serological assays on supernatant fluids obtained before and after inactivation indicated that neither the phenol nor the 18 hour period of inactivation had any measurable effect on the suitability of the supernatants for the additional processing required to obtain the purified polysaccharide. Accordingly, thirty liters of clear supernatant fluids derived from 72 hour cultures of P. aeruginosa were provided to the Department of Bacterial Diseases for further processing and purification.

3. Dengue Virus Vaccines.

During the past year studies were continued on the development of a dengue virus type 2 (DEN-2) vaccine for human use. In addition, attempts were made to obtain a temperature sensitive dengue virus type 3 (DEN-3) seed strain by passaging and cloning.

a. The S-1 DEN-2 virus from the lot #2 vaccine (Eckels, K.H. et al, 1976) was passaged further in certified fetal rhesus monkey lung (DBS-FRhL-2) cells at 31° C in order to determine if the virus would undergo further attenuatio n in terms of temperature sensitivity and pathogenicity for suckling mice.

For the first passage, confluent monolayers of DBS-FRhL-2 cells in 25 cm^2 flasks were inoculated with 0.2ml of a 10^{-3} dilution of the vaccine. Virus was adsorbed at 31° C for 90 minutes; maintenance medium (F-15 Eagle's Minimum Essential Medium, 2% Fetal Bovine Serum, 0.1% Streptomycin and Neomycin) was added and the flasks were than incubated at 31° C. On day 4, the flasks were refed with the same maintenance medium and returned to 31° C for an additional 3 days. On day 7, the flasks were harvested and the fluids mixed with an equal volume of inactivated Fetal Bovine Serum, (FBS), frozen and stored at -70° C.

For the second passage, the day 7 harvest from the first passage was diluted, inoculated and harvested using the same procedures as above.

Initially, the third passage was also done using the same procedures as previously indicated. The day 7 harvests, however, had been continually dropping in infectious titer. Therefore, the third passage was repeated using the same procedures except that the day 7 harvest from the second passage was diluted to 10^{-2} instead of 10^{-3}. All further passages were done in the same way. The results of the ten additional passages of the S-1 DEN-2

virus are given in the following Table XIV.

Table XIV

Passages of DEN-2 (PR 159) S-1 Lot #2
Vaccine Virus in DBS-FRhL-2 Cells at 31°C

Passage	Day of Harvest	Titer (PFU/0.2ml)
1	4*	2.0×10^1
	7	2.3×10^4
2	4*	$<10^1$
	7	5.2×10^3
3	4*	3.3×10^2
	7	5.8×10^4
4	4*	1.2×10^2
	7	2.2×10^4
5	4*	1.7×10^2
	7	4.5×10^4
6	4*	1.4×10^3
	7	1.3×10^5
7	4*	1.4×10^2
	7	3.1×10^4
8	4*	2.8×10^3
	7	1.9×10^5
9	4*	4.4×10^3
	7	1.5×10^5
10	4*	1.4×10^4
	7	5.6×10^5

* Culture fluids harvested and cell sheets refed with EMEM+2% FBS

By the tenth passage, the small plaque characteristic of the S-1 virus had changed into a mixed population of large and small plaques. However, when the same virus was plaqued by another plaquing system, (K. Eckels, Dept Hazardous Microbiology, WRAIR), the S-1 virus retained its small plaque morphology. (The only difference between the two plaquing systems is that Dr. Eckels' uses Noble agar plus DEAE-dextran in place of agarose and no DEAE-dextran in our system).

The virus population of some of the passages was also periodically checked for temperature sensitivity at 39.3°C and no plaques were formed at that temperature.

Selected passages were then assayed for temperature sensitivity at 38.5°C. The results are presented in Table XV.

Table XV

Temperature Sensitivity of Selected Passages
of the DEN-2 S-1 Lot #2 Vaccine Virus

Passage	35°C	38.5°C	EOP*
1	1.1×10^4	1.5×10^2	.0136
2	4.6×10^3	<1	.0002
4	2.2×10^4	1.3×10^2	.0059
6	5.0×10^3	3.5×10^1	.0070
8	8.0×10^4	1.3×10^1	.0002
10	4.1×10^5	1.6×10^4	.0390
S-1 Lot #2	1.3×10^5	2.8×10^2	.0022

* EOP - Efficiency of Plating = $\frac{38.5°C}{35°C}$

Passage of the S-1 virus at 31°C did not show a consistent trend which would indicate a definite increase in its temperature sensitivity. In fact, the virus from the tenth passage is approximately seventeen fold less sensitive than the S-1 virus as demonstrated by the EOP values in the above table. The pathogenicities of the selected passages are currently being determined in suckling mice.

b. The initiation of the passage of DEN-2 virus in chick fibroblasts was previously described (Annual Report, 1977), at which time the virus had to be passed into embryonated chick eggs by the intravenous route prior to chick fibroblasts. During this year another method was found to be just as successful and eliminated the inoculation of embryonated chick eggs. This involved the feeding of the chick fibroblast monolayers on a daily basis.

Confluent monolayers of chick fibroblasts in 25 cm^2 flasks were inoculated with 0.5 ml of seed material. After the virus was adsorbed at 35°C for 1 hour, the flasks were refed with maintenance medium (M-199, 2% FBS and 0.1% S&N). The flasks were then harvested and fed with maintenance medium on a daily basis. The harvested fluid that had the highest infectious titer was then used for the next passage. The first five passages of DEN-2 virus in chick fibroblasts are presented in the next table.

Table XVI

Passage of DEN-2 Virus in Chick Fibroblasts

Passage	Day of Harvest	Titer (PFU/0.2ml)
1	6	3.6×10^1
2	3	1.5×10^3
3	3	2.3×10^2
4	3	3.4×10^2
5	3	1.2×10^3

Further passages will be done in chick fibroblast cultures in an attempt to increase the yield of infectious virus.

c. It was previously demonstrated (Annual Report, 1977) that three doses of an inactivated DEN-2 vaccine were required for significant serological conversions in animals. This requirement of three doses may be due to the fact that the antigenic mass of the vaccine is not sufficient to elicit antibodies in fewer doses. After numerous attempts, the only method that increased the antigenic mass of the vaccine involved precipitating the virus from the culture fluid. At the present time, however, this method does not appear to be economically feasible.

Another factor that may contribute to the requirement of three doses of vaccine is the method employed in the inactivation of the virus. Previous experimental DEN-2 vaccines were inactivated with 0.05% formalin (HCHO) at 22°C for 6 days. Therefore, the following experiment was performed to determine the effect of different concentrations of formalin at various temperatures on the inactivation of DEN-2 virus.

Confluent monolayers of DBS-FRhL-2 cells in 150 cm^2 flasks were washed with 25 ml of Hank's Balanced Salt Solution (HBSS) and then inoculated with DEN-2 seed material (Annual Report, 1974) at a multiplicity of infection (MOI) of 1. Virus was adsorbed at 35°C for 90 minutes, followed by removal of the inoculum and three rinses of the cell monolayers with 25 ml of HBSS. After the addition of 50 ml of maintenance medium (Eagle's Minimum Essential Medium with antibiotics but no serum additives), the flasks were incubated at 35°C. On the basis of hemagglutination (HA) titer, the culture fluids from infected cell monolayers were collected, centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant passed through a 0.45μm membrane filter. This filtered material was pooled and 2% Human Serum Albumin (HSA) was added. This pooled material was divided into five 50 ml portions for each temperature (4°C, 22°C, and 35°C). Four

portions received different concentrations of formalin (0.025%, 0.05%, 0.1%, and 0.2%) and the fifth portion had no formalin. The inactivating fluids were held at the various temperatures and sampled at 6, 12, 24, 48, 72, 96, and 120 hours. A 1 ml sample from each portion was removed, mixed with an equal volume of inactivated fetal bovine serum (FBS), frozen, and stored at -70 C until assayed by plaquing in LLC-MK_2 cell monolayers. Previous experiments (Annual Report, 1976) have demonstrated that the 0.2% formalin sample had a detrimental effect on the LLC-MK_2 cells of the plaquing system, therefore, another sample from each portion was neutralized with the required amount of 1% sodium bisulfite ($NaHSO_4$) and stored at -70 C using the same procedures as above. The results of the inactivation of the virus for all samples are summarized in Table XVII.

Table XVII

The Effect of Temperature and Formalin on the Inactivation of DEN-2 Virus:

Titers of Non-Neutralized Formalin Samples

Temp.	Time (hrs.)	Formalin Concentration 0.0%	0.025%	0.05%	0.1%	0.2%
4°C	6	$1.3x10^6$*	$2.9x10^5$	$1.6x10^5$	$4.1x10^4$	$1.0x10^3$
	12	$1.2x10^6$	$1.9x10^5$	$8.4x10^4$	$1.7x10^4$	$2.3x10^2$
	24	$8.3x10^5$	$1.7x10^5$	$2.0x10^4$	$1.7x10^3$	$<10^1$
	48	$9.2x10^5$	$3.6x10^4$	$4.0x10^3$	$1.4x10^2$	$<10^1$
	72	$1.0x10^6$	$1.4x10^4$	$8.0x10^2$	$1.5x10^1$	$<10^1$
	96	$9.2x10^5$	$9.1x10^3$	$2.6x10^2$	$1.0x10^0$	$<10^1$
	120	$7.8x10^5$	$3.0x10^3$	$1.3x10^2$	0	-
22°C	6	$1.0x10^6$	$5.2x10^4$	$5.2x10^3$	$9.0x10^1$	$<10^1$
	12	$7.6x10^5$	$6.5x10^3$	$1.2x10^2$	0	$<10^1$
	24	$1.0x10^6$	$7.5x10^2$	$1.0x10^1$	0	$<10^1$
	48	$6.1x10^5$	$2.5x10^1$	0	0	$<10^1$
	72	$4.4x10^5$	0	0	0	ND
	96	$2.9x10^5$	0	0	ND	ND
	120	$2.0x10^5$	0	0	ND	ND
35°C	6	$8.6x10^5$	$5.5x10^2$	$1.5x10^0$	0	$<10^1$
	12	$3.8x10^5$	$1.5x10^1$	0	0	$<10^1$
	24	$1.4x10^5$	0	0	0	$<10^1$
	48	$2.5x10^4$	0	0	ND	ND
	72	$3.8x10^3$	0	0	ND	ND
	96	$1.5x10^3$	0	0	ND	ND
	120	$6.0x10^1$	0	0	ND	ND

*PFU/0.2 ml

Table XVII (con'd)

The Effect of Temperature and Formalin on the Inactivation of DEN-2 Virus:

Titers of Neutralized Formalin Samples

Temp.	Time (hrs.)	Formalin Concentration 0.0%	0.025%	0.05%	0.1%	0.2%
4 C	6	ND	4.2×10^5*	2.2×10^5	4.1×10^4	2.0×10^2
	12	ND	1.5×10^5	6.6×10^4	8.3×10^3	2.5×10^1
	24	ND	2.9×10^5	6.4×10^4	1.6×10^3	0
	48	ND	4.2×10^4	3.3×10^3	1.4×10^2	0
	72	ND	2.1×10^4	1.5×10^3	1.0×10^1	0
	96	ND	7.9×10^3	3.5×10^2	0	0
	120	ND	4.5×10^3	1.6×10^2	0	0
22 C	6	ND	5.3×10^4	5.6×10^3	4.0×10^1	3.0×10^1
	12	ND	5.8×10^3	1.1×10^2	0	0
	24	ND	1.1×10^3	1.0×10^1	0	0
	48	ND	3.5×10^1	0	0	0
	72	ND	0	0	0	ND
	96	ND	0	0	ND	ND
	120	ND	0	0	ND	ND
35 C	6	ND	2.0×10^1	2.0×10^1	3.0×10^0	1.0×10^0
	12	ND	1.0×10^0	0	0	0
	24	ND	0	0	0	0
	48	ND	0	0	ND	ND
	72	ND	0	0	ND	ND
	96	ND	0	0	ND	ND
	120	ND	0	0	ND	ND

*PFU/0.2 ml

As can be seen from the above table, there is a good correlation between the infectious titers of the neutralized and non-neutralized samples. On the basis of these results, a maximum of two days inactivation was selected for the production of a DEN-2 vaccine. Five vaccines were made in which the temperatures and the concentrations of formalin consisted of the following:

1. 4°C, 0.2% HCHO
2. 22°C, 0.05% HCHO
3. 22°C, 0.1% HCHO
4. 35°C, 0.025% HCHO
5. 35°C, 0.2% HCHO

The fifth vaccine was included in this study to determine the effect, if any, of a large amount of formalin, on the immunogenicity of the virus in terms of animal protection. The inactivation procedure using 0.2% formalin at 35°C was chosen as the one most likely to demonstrate this effect.

Mice (weanlings to four weeks old) were immunized with varying doses of each vaccine to re-evaluate the number of doses required for protection. For each vaccine, 10 mice received 0.5 ml intraperitoneally (IP) on day 0, 10 other mice received 0.5 ml on days 0 and 7, and 10 mice received three doses each of undilute, 1/5 and 1/25 on days 0, 7 and 21. All mice were challenged on day 28 with WP 130 strain of DEN-2 virus (Annual Report, 1977). The results are presented in Table XVIII.

Table XVIII

Mouse Potency Assay of Various Vaccines
Using Different Inactivation Procedures

	Inactivation Procedure				
	35°C, 0.025% HCHO	35°C, 0.2% HCHO	22°C, 0.05% HCHO	22°C, 0.1% HCHO	4°C, 0.2% HCHO
1 Dose:					
Undil.	30*	40	0	40	30
2 Doses:					
Undil.	40	20	80	30	40
3 Doses:					
Undil.	78	63	71	46	78
1/5	0	40	20	40	71
1/25	29	20	13	30	0

All mice challenged IC on Day 28 with 480 LD_{50} doses of WP 130 strain of DEN-2

* Percent of survival

These results substantiate the previous observation that three doses of vaccine are required for maximum protection.

Two strains of DEN-2 virus, WP 130 and PR 159, were also evaluated for immunogenicity in terms of a mouse potency assay. Vaccines from each strain using the 0.05% formalin, 22°C inactivation procedure were produced by previously indicated procedures. At the same time the effect of neutralization of the formalin with sodium bisulfite immediately after the required time of inactivation was compared to neutralization of the formalin just prior to immunization of the mice. The results are in the following table.

Table XIX

Comparison of Vaccines Prepared from Two Strains of DEN-2 Virus in the Mouse Potency Assay

3 Doses:	WP 130	WP 130 N**	PR 159	PR 159 N
Und.	56*	88	50	60
1/5	75	60	43	20
1/25	33	10	40	25

* Percent of survival
**N=formalin neutralized immediately after the required time of inactivation.

All mice challenged IC on Day 28 with 380 LD_{50} doses of WP 130 strain

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The vaccine made from the WP 130 strain of DEN-2 virus appears to be more protective than the vaccine made from the PR 159 strain. However, a number of mice died from anaphylactic shock. Studies will be conducted to attempt to eliminate the deaths due to anaphylactic shock, which is most probably caused by the HSA in the vaccine.

d. Dengue virus type 3, strain CH 53489 (DEN-3) had undergone eleven passages in the certified DBS-FRhL-2 cells (Annual Report, 1977). A selected number of these passages were assayed for temperature sensitivity at 39.3°C in the LLC-MK_2 plaquing system. The results of this study are presented in Table XX.

Table XX

Temperature Sensitivity of Selected Passages of DEN-3

Passage	35°C	39.3°C	EOP*
3	3.0×10^3	7	.0023
5	1.6×10^3	3.1×10^1	.0194
7	2.3×10^3	5.7×10^1	.0248
9	3.7×10^3	1.7×10^1	.0046
10	2.9×10^4	6	.0002

*EOP - Efficiency of Plating - $\frac{39.3^\circ C}{35^\circ C}$

From the above results, it appears that the temperature sensitivity of the virus may be increasing in the higher passages.

Since a plaque assay of DEN-3 virus in these certified cells is not yet possible, the passage 11 material was cloned three successive times by a terminal dilution technique in Linbro plates. Three separate clones were passaged through this terminal dilution technique, thus constituting passages 12, 13 and 14. Two additional passages in certified DBS-FRhL-2 cells were done in order to increase the titer of each clone. The following table shows the results of these two passages.

Table XXI

Passages of Different Clones of DEN-3 Virus
in Certified DBS-FRhL-2 Cells

Clone	Passage	Day of Harvest	Titer (PFU/0.2 ml)
C4B4C4	15	3*	2.0×10^1
		7*	1.5×10^3
		10	1.9×10^3
B5C1D6	15	3*	2.5×10^0
		7*	7.5×10^2
		10	1.8×10^3
C2D5C5	15	3*	1.0×10^0
		7*	4.7×10^1
		10	7.0×10^2
C4B4C4	16	3*	7.5×10^0
		7*	1.7×10^3
		10	3.4×10^3
B5C1D6	16	3*	6.0×10^0
		7*	1.6×10^3
		10	3.4×10^3
C2D5C5	16	3*	0
		7*	7.5×10^2
		10	1.6×10^3

*Culture fluids harvested and cell sheets refed with EMEM + 2% FBS

At passage 16, a small pool of each clone was made for further studies.

Growth curves in DBS-FRhL-2 cells were done for each clone at various temperatures. Confluent monolayers of DBS-FRhL-2 cells in 25 cm^2 flasks were inoculated with 0.2 ml of passage 16 from each clone. Virus was adsorbed at 33°C for 90 minutes. After the addition of 5 ml of maintenance medium (F-15 EMEM, 2% FBS and 0.1% S&N), the flasks were incubated at the various temperatures. At 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days post-inoculation, a 0.1 ml sample from each flask was removed, mixed with 0.9 ml diluent (EMEM + 1% HSA) and frozen at -60°C. On days 4 & 7 the flasks were refed with maintenance medium after the 0.1 ml sample was removed. Samples were assayed by plaquing in LLC-MK_2 cell monolayers. The results of this experiment are shown in Table XXII.

Table XXII

Growth Curves of Each DEN-3 Virus Clone
at Various Temperatures

Days	C4B4C4	B5C1D6	C2D5C5
		33°C	
1	2.0×10^1*	2.0×10^1	1.5×10^1
2	1.8×10^2	8.5×10^1	$<10^1$
3	1.1×10^3	9.3×10^2	$<10^1$
4	2.7×10^3	2.5×10^3	5.4×10^2
5	5.1×10^3	5.2×10^3	9.1×10^2
6	8.2×10^3	8.5×10^3	2.9×10^3
7	9.4×10^3	1.1×10^4	3.5×10^3
8	4.7×10^4	3.5×10^4	2.3×10^4
9	3.2×10^4	2.2×10^4	1.4×10^4
10	1.3×10^4	1.3×10^4	8.0×10^3
		37°C	
1	$<10^1$	$<10^1$	$<10^1$
2	9.5×10^1	8.0×10^1	1.0×10^1
3	5.8×10^2	9.3×10^2	4.0×10^1
4	4.9×10^2	7.5×10^2	8.0×10^1
5	1.7×10^3	2.4×10^3	3.2×10^2
6	3.8×10^3	4.1×10^3	4.7×10^2
7	2.2×10^3	3.5×10^3	4.1×10^2
8	4.4×10^4	1.2×10^4	1.9×10^3
9	9.0×10^3	7.5×10^3	2.7×10^3
10	5.9×10^3	7.0×10^3	1.8×10^3
		38.5°C	
1	$<10^1$	$<10^1$	$<10^1$
2	1.0×10^1	$<10^1$	$<10^1$
3	4.5×10^1	4.5×10^1	$<10^1$
4	3.5×10^1	1.0×10^2	$<10^1$
5	2.8×10^2	5.8×10^2	1.0×10^1
6	5.9×10^2	7.3×10^2	2.0×10^1
7	4.3×10^2	7.7×10^2	1.5×10^1
8	2.6×10^3	3.9×10^3	1.2×10^2
9	1.3×10^3	2.4×10^3	1.8×10^2
10	1.0×10^3	1.1×10^3	7.5×10^1

*PFU/0.2 ml

The only clone that appeared to be somewhat temperature sensitive at 38.5 C°was the C2D5C5 clone. This clone also showed a slight decrease in titer at 37°C. The other two clones showed basically no differences in titer at 33°C or 37°C and only a slight decrease at 38.5°C.

Since C2D5C5 clone was the only one that showed any evidence of temperature sensitivity, this clone was further characterized by doing a growth curve in sucklin mouse brains. Suckling mice were inoculated intracerebrally (IC) with 0.02 ml of the C2D5C5 clone and two mice were removed daily for 16 days, killed and frozen whole. On the day of assay the mice were thawed in a water bath and the brains were removed with a syringe. The brain materials were diluted with diluent (EMEM + 1% HSA) to make 20% suspensions and spun at 2000 rpm for 20 minutes. The supernatants were assayed in the LLC-MK_2 plaquing system. The results are summarized in the following table.

Table XXIII

Growth Curve of C2D5C5 Clone in Suckling Mouse Brain

Day	Titer (PFU/0.2 ml)
1	$<10^1$
2	$<10^1$
3	$<10^1$
4	3.9×10^2
5	2.2×10^2
6	9.2×10^2
7	6.5×10^3
8	6.9×10^3
9	2.2×10^4
10	3.1×10^3
11	5.6×10^2
12	1.1×10^2
13	9.0×10^1
14	1.5×10^1
15	$<10^1$
16	$<10^1$

As can be seen from the above table, the C2D5C5 clone replicated fairly well in the brains of suckling mice. Thus this clone does not appear to be a potential candidate vaccine seed strain, especially since a clone isolated by Dr. Eckels (Dept Hazardous Microbiology, WRAIR) was more temperature sensitive and did not replicate to any extent in the brains of suckling mice. Therefore, work on this clone was terminated.

4. Diagnostic Reagents.

As part of a continuing effort to improve the stability and reduce the logistic requirements of diagnostic reagents required by military laboratories, a study was initiated on the feasibility of preparing stable, freeze-dried diagnostic antisera (for slide agglutination tests) in pre-packaged single-test units. These would be rehydrated and used in situ only as required for the number of tests actually to be accomplished in a laboratory, in place of the present method which involves the withdrawing of the volumes needed from a multi-test container, and distribution to a series of slides for use.

A satisfactory single-test container was found to be the clear, plastic 96-well Linbro Disposo-Tray (Model 96SC-12 wells x 8 wells). Six diagnostic antisera, currently prepared and distributed by this laboratory, were selected for testing. For each of the six preparations on a single tray, a drop of antiserum was dispensed with a 1 ml pipette into the eight wells in a single row. The rows of wells between the serum wells were filled with drops of normal serum (to serve as controls). The Linbro trays were then placed on the shelf of a -60°C mechanical freezer and then the preparations were freeze-dried in a chamber dryer. After drying, one set of trays was placed under vacuum in a vacuum jar and the wells of a second set were sealed with "Scotch" tape to keep dust and moisture out. All trays containing the freeze-dried antisera, together with fluid samples of each of the six antisera, were stored at 4°C and samples of each were tested at three month intervals to determine their comparative stabilities. To perform the test with the freeze-dried samples in the wells, a row of wells containing each of the antisera and the normal serum controls, was cut from the trays with scissors. Each of the freeze-dried antisera and a normal serum for each were rehydrated and mixed with a drop of an appropriate suspension of the homologous 18 hour bacterial culture. The fluid preparations were tested by mixing a drop of serum with a drop of the homologous culture on a slide. Both wells and slides were held for two to three minute at room temperature and the degree of agglutination in each was then recorded. The results of tests performed after three, six and nine months storage of the freeze-dried preparations at 4°C are recorded in Table XXIV.

Table XXIV

Stability of Freeze-Dried Antisera in Disposo-Tray Wells

Antiserum	Months in Storage at 4 C			
	0	3	6	9
Salmonella typhosa "H"				
Fluid	4+	4+	4+	4+
Freeze-Dried - Under vacuum		4+	4+	4+
No vacuum	4+	4+	4+	4+
Salmonella schottmuelleri "H"				
Fluid	4+	4+	4+	4+
Freeze-Dried - Under vacuum		4+	4+	4+
No vacuum	4+	4+	4+	4+
Shigella boydii 5				
Fluid	4+	4+	4+	4+
Freeze-Dried - Under vacuum		4+	4+	4+
No vacuum	4+	4+	4+	4+
Vibrio comma "O"				
Fluid	4+	4+	4+	4+
Freeze-Dried - Under vacuum		2+	+	2+
No vacuum	2+	2+	+	2+
Escherichia coli				
Fluid	4+	4+	2+	4+
Freeze-Dried - Under vacuum		4+	2+	+
No vacuum	4+	4+	2+	+
Shigella flexneri 1				
Fluid	4+	4+	4+	4+
Freeze-Dried - Under vacuum		4+	2+	4+
No vacuum	4+	4+	4+	4+

The results indicate that, with the exception of the V. comma "O" antiserum, there was no apparent loss in agglutinability as the result of freeze-drying of the various preparations. In addition, all but the E. coli antiserum appeared stable over the nine month period of storage at 4°C. There also appears to be no advantage to storing the freeze-dried preparations under vacuum for the nine month period tested thus far. Stability tests on the antisera will be continued.

This pre-packaged freeze-dried diagnostic antiserum preparation appears to be a feasible way of providing an inexpensive and rapid method for performing agglutination tests. It reduces logistical requirements by eliminating the shipment of bottles of fluid antisera, slides, etc., which are now needed by diagnostic laboratories for performing these tests.

5. Vaccine Potency Assays.

During the past year studies have been resumed on the development of a mouse protection assay as an in vivo method for the evaluation of group B meningococcal vaccines.

Previous studies (Annual Report, 1974) have demonstrated that excellent protection was afforded Walter Reed ICR mice immunized with meningococcal protein vaccines and subsequently challenged with virulent Neisseria meningitidis, strain B-11, suspended in 5% hog gastric mucin. For these experiments Walter Reed ICR mice, weighing 13-16 gm, were used for immunization and the challenge dose consisted of approximately 3600 organisms, which was equivalent to approximately 180 LD_{50} doses. The interval between immunization and challenge was seven days. Preliminary sensitivity studies suggested that 4-fold differences in ED_{50} values were significant.

Studies in our department and in the Department of Bacterial Diseases, WRAIR, have resulted in the production of several protein-polysaccharide complex vaccines prepared from N. meningitidis, strain B-11, for possible use in humans. Thus, it was of considerable interest, using the mouse potency assay, to investigate the protective response of available vaccine lots in mice and to attempt to correlate the results with those found in humans.

Since these mouse studies were carried out, however, an infection in the Walter Reed ICR mouse colony necessitated the destruction of the colony and the establishment of a new ICR colony. Preliminary virulence titrations revealed that the newly formed ICR strain was considerably more resistant to meningococcal strain B-11 than was the original Walter Reed mouse strain. This was consistent with the increased resistance of these mice to infection with Salmonella typhi, strain Ty2.

Previous investigations (Annual Report, 1975) have shown that the addition of iron to substandard lots of mucin sufficiently enhanced the virulence of S. typhi, strain Ty2, to permit its use in mouse potency assays for the evaluation of typhoid vaccines.

Because of the increased resistance encountered with the new ICR mouse colony to meningococcus infection, it was considered desirable to determine whether the addition of iron to gastric mucin would have a virulence-enhancing effect on meningococcus strain B-11 in these mice.

Iron, in the form of ferric ammonium citrate, in increasing concentrations, was added to a 5% mucin suspension. Appropriate dilutions of N. meningitidis strain B-11 were prepared in the iron-mucin suspensions, and in mucin alone, and were injected into mice weighing 18-20 gm. The results of the titrations are shown in Table XXV.

Table XXV

The Effect of Iron on the Virulence
of N. meningitidis Strain B-11 for Mice

Amount of Iron (Fe^{+++}) Added to Mucin*	LD_{50} Value**			
	ICR Males		ICR Females	
No Iron	700	Organisms	360	Organisms
5 mg Fe^{+++}/kg mouse body weight	50	"	25	"
10 " " " " " "	7.0	"	1.7	"
20 " " " " " "	2.0	"	1.7	"

* Mucin Lot #0347A001, Wilson Laboratories
** Calculated by the Miller-Tainter probit method
Ferric Ammonium Citrate used as source of Fe^{+++}

The results demonstrated that the addition of iron to mucin suspensions did enhance the virulence of meningococcus strain B-11, and as the concentration of iron was increased, there was a further increase in virulence. Similar reduction in LD_{50} values were obtained when iron was added to one other mucin lot, #96181, which when tested in previous titrations was not as effective a virulence enhancer for strain B-11 as was mucin lot #0347A001.

The usefulness of mucin supplemented with iron in mouse potency assays for meningococcal vaccines will be further evaluated. Mouse potency assays on protein-polysaccharide complex vaccines will be performed to correlate the protection afforded mice with results obtained from human immunization.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 136 Development of Biological Products

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References.

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Publications.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6537	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NI	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	137
b. CONTRIBUTING				
XXXXXXXXX	Cards 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pathologic Manifestations of Zoonotic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 02	Cont.	DA	C. In House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	8	212
FISCAL YEAR CURRENT 79	8	16

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

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NAME: KEENAN, K.P., BUNTE, R.

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Wildlife; (U) Kidney; (U) Protozoa
(U) Electron Microscopy; (U) Intravascular Coagulation; (U) Immunopathology; (U) Brain

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To study and define pathogenesis of spontaneous and experimental trypanosomiasis, leishmaniasis and sarcosporidiosis in a variety of animal hosts. To study spontaneous severe renal disease and deaths in colonized and quarantined aotus monkeys. Provide morphologic pathology support for wildlife epidemiological surveys in the Trans Amazon and other military installations. Provide pathology support for spontaneous disease and deaths occurring in rodent colonies at WRAIR. All projects investigated are of military importance as a basis for the understanding of human diseases.

24(U) Studies will utilize conventional gross and histopathology, clinical pathology, histochemistry, immunohistochemistry and electron microscopic techniques.

25(U) 77 10-78 09 The investigative study of the role of disseminated intravascular coagulation in the pathogenesis of Trypanosoma rhodesiense infection in dogs and rats is being continued. Ultrastructural neuropathology studies of experimental Trypanosoma congolense infection in African cattle is being studied. A study of the pathology of chronic Trypanosoma rhodesiense infection in mice is in progress. The pathology and pathogenesis of experimental leishmaniasis in hamsters, mystromys and dogs are being studied. A study to define the pathogenesis of glomerulonephritis induced in cattle by Sarcocystis fusiformis is being investigated in collaboration with USDA Research Institute, Beltsville, MD. The susceptibility and evolution of Sarcocystis suihomonis in subhuman primates are being investigated. The pathology and pathogenesis of spontaneous disease with severe renal injury in the aotus monkey are being studied. The pathology of IA-4-N-oxide treated Schistosoma mansoni infected rhesus monkeys is completed. Anatomic lesions which were identified and statistical data compiled on wildcaught animals as part of a large epidemiological survey is being continued in the second phase. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Investigators
Principal: Anthony J. Johnson, MAJ, VC
Associates: Michael J. Reardon, MAJ, VC, Ralph M. Bunte, MAJ, VC, Kevin P. Keenan, CPT, VC, John C. Seely, CPT, VC, Carol A. Thompson, CPT, VC, LuAnn McKinney, CPT, VC, Charlotte A. Miller, CPT, VC, and Henry G. Wall, MAJ, VC

Background:

To diagnose, define, investigate, compare and analyze known and potential diseases common to man and animals. Special emphasis will be focused on control and protective methods against the occurrence and tissue injury induced by these diseases. A major effort will be directed toward defining the mechanistic events and reactions at the cellular and subcellular levels occurring in the development of these diseases. Gross pathology, histopathology, clinical pathology, ultrastructural pathology, histochemistry, and immunohistochemistry will be used.

During the reporting period the following research activities have been in progress: 1) Pathogenesis of Trypanosoma rhodesiense infection in C4-deficient guinea pigs and those with a normal complement system; 2) Pathogenesis of Trypanosoma rhodesiense infection in dogs; 3) The role of disseminated intravascular coagulation in the pathogenesis of Trypanosoma rhodesiense in rats; 4) Pathology of chronic Trypanosoma rhodesiense infection in mice (strain C57BL/6J); 5) Electron microscopic study of central nervous system and renal injuries in cattle experimentally infected with Trypanosoma congolense; (6) Mystromys albicaudatus as a laboratory animal model of South American cutaneous leishmaniasis; 7) Pathology of Leishmania mexicana infection in the golden hamster; 8: Pathology of experimental Leishmania donovani (Khartoum) infection in the cynomolgus monkey; (9) Electron microscopic examination of forms of Leishmania brasiliensis derived from cultivation in various axenic media; (10) Pathogenesis of glomerulonephritis induced in cattle by Sarcocystis cruzi; (11) Transmission of Sarcocystis suihomonis from humans to swine to nonhuman primates; (12) Pathology of IA-4-N-oxide treated Schistosoma mansoni infected rhesus monkeys; (13) Effects of increased dietary fibers on zinc and magnesium levels in human vol-

unteers; (14) Clinical pathology studies of the aotus monkey; (15) Etiology of spontaneous glomerulonephritis in aotus monkey; (16) Pathology in rats with temporary loss of the micturation reflex after destruction of areas in the region of the locus coeruleus; (17) Pathology of spontaneous coronavirus infection in dogs; (18) Investigation of thymic necrosis in neonatal mice; (19) Histopathology of activity-stress ulcers in rats; (20) Experimental investigation of nephrotoxic effects of Diatriazoate in the rat following ligation of the common bile duct; (21) TransAmazon epidemiological survey; (22) Pathology of rodents in a surveillance program; and (23) Respiratory disease in random source canines.

1. Pathology Of Trypanosoma rhodesiense Infection In C4-Deficient Guinea Pigs And Those With A Normal Complement System.

This study was summarized in the last annual report. The data has been analyzed and assembled into two draft manuscripts. Further studies on this model are not planned.

2. Pathogenesis Of Trypanosoma rhodesiense In Dogs.

This study was conducted to evaluate the gross pathology, histology, clinical pathology, immunopathology and ultrastructural pathology of Trypanosoma rhodesiense infection in the dog and its pathogenesis.

In Phase II (chronic infection studies), it was determined that a true chronic disease could not be established with the strain of Trypanosoma rhodesiense used. However, the additional data obtained indicated an active but inadequate humoral immune response.

Both Phase I and Phase II data results are being analyzed for project completion.

3. The Role Of Disseminated Intravascular Coagulation In The Pathogenesis Of Trypanosoma rhodesiense Infection In The Rat.

In collaboration with the Department of Immunology, WRAIR.

The effect of experimental trypanosomiasis on coagulation is being assessed because human patients with Trypanosoma rhodesiense infection are reported to have developed thrombocytopenia with disseminated intravascular coagulation.

Rats injected intraperitoneally with Trypanosoma rhodesiense developed acute, severe trypanosomiasis and thrombocytopenia with-

out significant changes in RBC counts or fibrinogen and fibrin split product levels. In this study it was observed that the complement system is altered during the course of the infection and evidence of immune complex deposits could be demonstrated in the kidneys. Correlation between the thrombocytopenia and parasitemia was made.

This study is being continued into a second phase where the coagulopathy will be analyzed in the chronic disease.

4. Pathology Of Chronic Trypanosoma rhodesiense Infection In Mice (strain C57BL/6J).

In collaboration with the Department of Immunology, WRAIR.

The need for a laboratory rodent model to study chronic Trypanosoma rhodesiense infection is well-recognized and well-publicized. Recently it was determined by investigators in the Department of Immunology, WRAIR, that strain C57BL/6J mice developed chronic trypanosomiasis when exposed to Trypanosoma rhodesiense.

The current study employing light microscopy, immunofluorescence microscopy, clinical pathology, histochemical and immunohistochemical techniques, and immunoelectrophoresis to evaluate this chronic infection is in progress. Special emphasis is being devoted to the renal injury and the brain lesions which usually develop late in the evolution of the disease in man.

5. Electron Microscopic Study Of Central Nervous System And Renal Injuries In Cattle Experimentally Infected With Trypanosoma congolense.

In collaboration with the WRAIR Medical Research Unit - Kenya.

African trypanosomiasis of domesticated cattle is characterized by irregular fever, anemia, weight loss, subcutaneous edema, weakness, and death. Death may follow an acute illness or after a prolonged course during which insiduous body wasting and weakness are the major features. The causative agents in these trypanosomal infections have been Trypanosoma congolense, T. vivax, and T. brucei. Trypanosoma brucei is the type species for T. rhodesiense and T. gambiense, the causative agents of trypanosomiasis in man. It has been confirmed that T. rhodesiense occurs in cattle and is associated with disease in this species. The biologic behavior of T. congolense differs from that of T. rhodesiense in the bovine host and hence the pathogenesis of the lesions induced by these respective trypanosomes may differ. By

light microscopy a definite difference can be recognized in the brain lesions induced by T. congolense and T. rhodesiense.

The purpose of this study is to analyze the pathogenesis of T. congolense induced brain and kidney injuries and subsequently compare this with the pathogenesis of injury induced in these organs by T. rhodesiense. Light microscopy, electron microscopy, immunofluorescence microscopy, histochemistry, and immunohistochemistry techniques will be utilized to make the comparison.

This study is being continued.

6. Mystromys albicaudatus As A Laboratory Model Of South American Cutaneous Leishmaniasis.

In collaboration with the Department of Parasitic Diseases, WRAIR.

A sequential evaluation (12 weeks) of the cutaneous lesion induced by Leishmania braziliensis panamanensis in the Mystromys rat has been completed. The initial raised lesion at the site of inoculation increased in size and ulcerated centrally by 3 or 4 weeks post-infection to become a severe focal ulcerative dermatitis. Secondary infection of the ulcer by a variety of bacteria was common. Through 12 weeks post-infection, the ulcer increased in size to approximately 2.0 centimeters in diameter in a majority of the rats. Microscopically, the fully developed cratiform ulcer had necrosis of epithelial cells, marked proliferation of histocytes and infiltration of lymphoplasmacytic cells at the periphery. Myriads of leishmanial organisms were observed in histocytes. The development and morphologic character of this leishmanial induced lesion in the Mystromys albicaudatus closely mimic the morphogenesis of the acute cutaneous lesion caused by this organism in man. To better characterize the model, additional studies are planned to evaluate chronic effects of L. braziliense in the Mystromys.

7. Pathology Of Leishmania mexicana Infection In The Golden Hamster.

The histopathology of Leishmania mexicana infection in the golden hamster will be compared with similar infections by L. tropica, L. chagasi, L. donovani and L. braziliensis in this rodent.

This study is currently in progress.

8. Pathology Of Experimental Leishmania donovani (Khartoum) Infection In The Cynomolgus Monkey.

In collaboration with the Department of Parasitic Diseases and

the Department of Biology, WRAIR.

This study was summarized in the last annual report. The data has been analyzed and assembled into two draft manuscripts. Further studies on this model are not planned.

9. Electron Microscopic Examination Of Leishmania braziliensis Forms Derived From Cultivation In Various Axenic Media.

In collaboration with the Department of Parasitic Diseases, WRAIR.

Human isolates of L. braziliensis were cultivated as both promastigotes and amastigotes in axenic media. The ultrastructural morphology of the forms were defined. In addition, amastigotes in infected macrophages and Vero cells were examined ultrastructurally and compared with the axenic-cultured amastigotes. This study had demonstrated that promastigotes and amastigotes from axenic media were surrounded by a single membrane, and furthermore, that axenic amastigotes resembled those in macrophages and Vero cells. It is currently speculated that the double membrane previously reported for amastigotes in macrophages is of host cell origin.

The data are currently being assembled in manuscript form.

10. Pathogenesis Of Glomerulonephritis Induced In Cattle By Sarcocystis cruzi.

This represents a collaborative study between the department and the Animal Parasite Institute, USDA, Beltsville, MD. This study and similar investigations were initiated upon request to this department by the Animal Parasitic Institute.

The study is designed to define the mechanistic causation of the glomerular injury induced by the Sarcosystis infection. Difficulty in analyzing the bovine complement has been encountered.

11. Transmission Of Sarcocystis suihomonis From Humans To Swine To Nonhuman Primates.

In collaboration with the Animal Parasite Institute, USDA.

Sarcocystis suihomonis is a two vertebrate host protozoan parasite that infect man and swine. In man it causes an intestinal infection that may be accompanied by diarrhea, vomition, nausea, and painful gastrointestinal upset. In swine this protozoan undergoes cyclopropagative development within endothelial and reticular cells and cysts in skeletal and cardiac musculature to remain dormant until the meat is consumed.

out significant changes in RBC counts or fibrinogen and fibrin split product levels. In this study it was observed that the complement system is altered during the course of the infection and evidence of immune complex deposits could be demonstrated in the kidneys. Correlation between the thrombocytopenia and parasitemia was made.

This study is being continued into a second phase where the coagulopathy will be analyzed in the chronic disease.

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the Department of Biology, WRAIR.

This study was summarized in the last annual report. The data has been analyzed and assembled into two draft manuscripts. Further studies on this model are not planned.

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Preliminary studies on the use of nonhuman primates as an experimental model to study this protozoan parasite have been completed. A paper is being assembled for publication.

12. Pathology Of IA-4-N-Oxide Treated Schistosome mansoni Infected Rhesus Monkeys.

In collaboration with the Department of Parasitology, WRAIR.

Phase III is completed and the data is being analyzed.

There are no current plans for further efficacy testing of this group of drugs in the rhesus monkey.

13. Effects Of Increased Dietary Fibers On Zinc And Magnesium Levels In Human Volunteers.

Analysis of data is complete and the findings have been reported at nutrition conferences in Canada and Brazil. No further studies are planned.

14. Clinical Pathology Studies Of The Aotus Monkey.

The results of hematological and serum chemistry screening of the Aotus colony indicates that a red blood cell count of 4.0×10^6/cm, an albumin concentration of 4.0 gm/dl, a blood urea nitrogen concentration of 25.0 mg/dl, and a creatinine concentration of 0.8 mg/dl are abnormal findings for this species. Using these criteria a point system has been devised awarding 1 point for each abnormal value. A score of 0 is perfect whereas an animal with a score of 4 is considered seriously ill. Although at present not statistically proven, the predictive value of this system appears to correlate well with death losses.

Serum electrophoretic studies are continuing and all adults in the colony have been examined. Juveniles are being sampled and their patterns compared to the parents. It appears that the serum protein electrophoretic profile is genetically controlled within specific karyotypes and several distinct types have been recorded. This data is being prepared for publication and has been presented at the National AALAS meeting in New York City, September 1978.

Further studies are being planned to correlate karyotype and malaria susceptibility by in vitro and in vivo methods.

15. Etiology Of Spontaneous Glomerulonephritis In Aotus Monkeys.

In collaboration with the Division of Veterinary Medicine,

WRAIR.

Gross and histopathologic examinations of Aotus monkeys dying spontaneously in the WRAIR colony continue to have a high incidence of moderate-to-severe mesangioproliferative glomerulonephritis. Light microscopic, ultrastructural, histochemical and immunohistochemical techniques indicate that the glomerulonephritis is associated with immune complex deposition in the glomeruli. Efforts to identify the etiologic agent(s) is being continued. A summary report of the current status of this investigation is being assembled.

16. Pathology In Rats With Temporary Loss Of The Micturation Reflex After Destruction Of Areas In The Region Of The Locus Coeruleus.

In collaboration with the Department of Microwave Research, WRAIR.

This preliminary study has been completed. Gross and light microscopic examinations were done on the carcasses and tissues of eleven affected rats. No anatomical changes were observed in the urinary tract which could be attributed directly to the destruction of areas in the region of the locus coeruleus. Only secondary changes associated with urine retention and self-inflicted trauma were seen. Our conclusions are that further, more sophisticated studies are needed to define the pathogenesis of the loss of the micturation reflex, and that the primary change is neurophysiological rather than anatomical.

17. Pathology Of Spontaneous Coronavirus Infection In Dogs.

In collaboration with the Division of Veterinary Medicine, WRAIR.

Binn, et al. (1974) recovered and characterized the first canine coronavirus isolated from fecal specimens of an epizootic of diarrheal disease in USAF military dogs in Germany. Keenan et al. (1976) studied the pathogenicity of the Binn's isolate and concluded that this coronavirus was mildly pathogenic and self-limiting.

A study is in progress to characterize and evaluate the pathogenicity of an apparently new strain of canine coronavirus isolated from the small intestine of two dogs which died spontaneously of enteritis and diarrhea in the WRAIR animal colony.

18. Investigation Of Thymic Necrosis In Neonatal Mice.

In collaboration with the Division of Veterinary Medicine, WRAIR.

This study was initiated to determine the cause(s) responsible for a natural outbreak of spontaneous deaths and thymic necrosis in 3 to 6 day old suckling mice. The thymic necrosis was seen in 13 of 14 litters examined during March and April 1977. Since that date no spontaneous thymic necrosis has been found in twenty selected litters of 3 to 6 day old mice. Efforts to initiate the disease by treating the mice with steroids have been unsuccessful. This study did indicate that the thymic necrosis and deaths were self-limiting in the colony. Should the disease and deaths recur efforts should be initiated to determine the cause. This study awaits a recurrence of the problem.

19. Histopathology Of Activity-Stress Ulcers In Rats.

In collaboration with the Division of Neuropsychiatry, WRAIR.

The glandular stomachs of these rats showed extensive pathology, ranging from mucosal hemorrhage, with or without focal necrosis, through acute ulcers characterized by discontinuity of the muscularis mucosa. With one exception, all experimental rats showed this entire range of pathology, and in addition, 33% had at least one lesion showing signs of regeneration. Focal erosions predominated, but all experimental rats had at least one ulcer penetrating the muscular mucosa.

These results have been published.

20. Experimental Investigation Of Nephrotoxic Effects Of Diatrizoate In The Rat Following Ligation Of The Common Bile Duct.

Tissues have been examined with the light microscope without knowledge of experimental treatment. These results have seen sent to the principal investigator for collation with clinical pathological and other experimental data.

21. TransAmazon Epidemiological Survey.

In collaboration with the USAMRU-Belem.

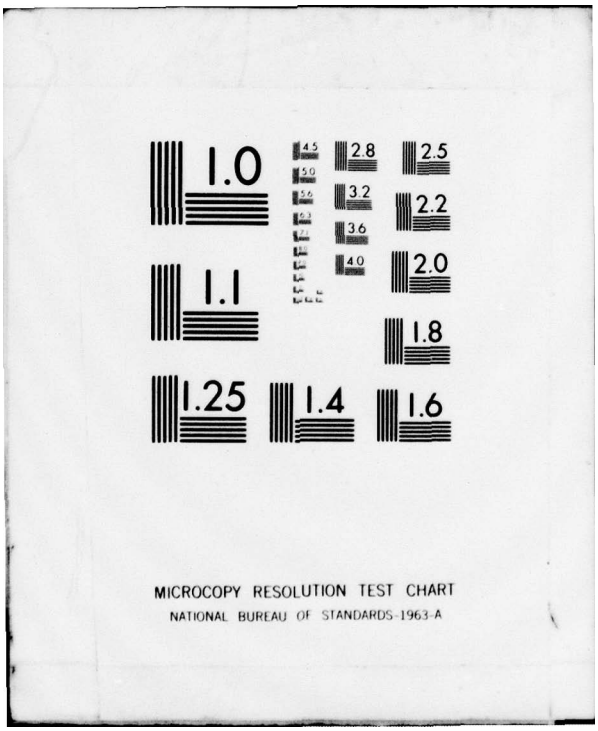
A report of the histopathologic lesions in 600 rodents, marsupials, bats and other miscellaneous mammals has been furnished the WRAIR Team-Belem. Additional tissues are to be submitted for histopathologic examination and analysis.

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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMIS--ETC(U)
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22. Pathology Of Rodents In A Surveillance Program.

In collaboration with the Division of Veterinary Medicine, WRAIR.

Gross and histopathologic examinations were performed on rats, mice and guinea pigs culled from breeding colonies at WRAIR. The data obtained from this ongoing study will be assembled for reporting and for maintaining a quality control record of the breeding colonies. This data assures the users of laboratory animals provided by WRAIR are of the highest quality.

23. Respiratory Disease In Random Source Canines.

In collaboration with the Division of Veterinary Medicine, WRAIR.

The objective of this study was to determine the cause of spontaneous respiratory disease in random source dogs housed at WRAIR. Gross and histopathologic findings were annotated and the pathogenesis of the respiratory changes were provided in a report format. Two papers of this study were presented at the 28th. annual meeting of AALAS. The papers have been submitted for publication in the AALAS journal.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Publications:

1. Binn, L. N., Marchwicki, R. H., Keenan, K. P., Strand, S. J., and Engler, W. F.: Recovery of reovirus type 2 from an immature dog with respiratory tract disease. Am. J. Vet. Res. 38: 927-929, 1977.

2. Binn, L. N., Alford, J. P., Marchwicki, R. H., Keefe, T. J., Beattie, R. J., and Wall, H. G.: studies of respiratory disease in random-source dogs: Viral infections in unconditioned dogs. Lab. Ani. Sci. (In press).

3. Fayer, R., Heydom, A. O., Johnson, A. J., and Leek, R. G.: Transmission of Sarcocystis suihomonis from humans to swine to nonhuman primates (Pan troglodytes, Macaca mulatta, Macaca irus). Z. Parasitenk. (In press).

4. Jacobson, E. R., and Seely, J. C.: Lymphosarcoma associated with viral-like intranuclear inclusions in a California king snake (Colubridae: Lampropeltis). J. Nat. Cancer Inst. (In press).

5. Jacobson, E. R., Seely, J. C., Novilla, M. N., and Davidson, J. P.: Heart failure associated with unusual hepatic inclusions in a Deckert's rat snake. J. Wildlife Dis. Assoc. (In press).

6. Keenan, K. P., Alexander, A. D., and Montgomery, C. A.: Pathogenesis of experimental Leptospira interrogans, serovar bataviae, infection in the dog: Microbiological, clinical, hematologic, and biochemical studies. Am. J. Vet. Res. 39: 449-454, 1978.

7. Keenan, K. P., Binn, L. N., and Takeuchi, A.: Animal model of human disease, acute nonbacterial gastroenteritis: Animal model: Acute enteritis in dogs infected with coronavirus 1-71. Am. J. Path., 1978 (In press).

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9. Manning, F. J., Wall, H. G., Montgomery, C. A., Simmons, C. J., and Sessions, G. R.: Microscopic examination of the activity-stress ulcer in the rat. Physiol. and Behav. 21: 269-274, 1978.

10. Reid, W. A., Loizeaux, P. S., and Reardon, M. J.: The activity of a benzothiopyranoindazole (IA-4-Oxide) against Schistosoma mansoni infection in Rhesus monkeys. Am. J. Trop. Med. and Hyg. 26: 937-943, 1977.

11. Seely, J. C.: Electron microscopy as a technique in diagnostic pathology. J. Wildlife Dis. Assoc. (In press).

12. Seely, J. C., Cosenza, S. F., and Montgomery, C. A.: Leiomyosarcoma of the canine urinary bladder with metastasis. J.A.V.M.A. 172: 1427-1429, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6530	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	138
b. CONTRIBUTING				
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Vaccine Development in Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	3	85
FISCAL YEAR CURRENT 79	3	85

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* HOCKMEYER, Wayne T. MAJ, MSC

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Muriithi, I. Dr.

NAME: Wellde, Bruce T.

21. GENERAL USE

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(U) Kenya; (U) Trypanosomiasis; (U) Vaccine; (U) Africa; (U) Cattle; (U) Immunity

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of this program is to develop an effective, practical vaccine against African trypanosomiasis, useful to both military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunity, host response and pathology of infection. There is a requirement for these studies which should provide a basis for rational development of a vaccine for this disease which would constitute a serious hazard for military personnel operating in the endemic area.
24. (U) Experiments conducted at WRAIR and in Kenya have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle, and monkeys can be rendered completely resistant to a challenging infection of T. rhodesiense. Partial immunity has been achieved against T. congolense.
25. (U) 77 10 - 78 09 During this period the investigators demonstrated that the antigenic character of the parasite population of T. rhodesiense from an endemic area was composed of perhaps as few as one serodeme which was antigenically stable over an 8-year period. They also found that immunity could be induced to blood and tsetse fly (metacyclic) forms by exposure of experimental animals to a broad spectrum of antigenic variants of the same serodeme. The sterile immunity was long lasting. It is believed that these findings enhance the likelihood of immunologic control of trypanosomiasis. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 October 1978-30 September 1979.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

PROJECT: 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES
WORK UNIT: 138 Vaccine Development in Trypanosomiasis
INVESTIGATORS: MAJ W.T.Hockmeyer, MAJ L.W. Roberts, B.T. Wellde, DAC

RESEARCH ON IMMUNIZATION AGAINST AFRICAN SLEEPING SICKNESS

A. INTRODUCTION

African trypanosomiasis is an important disease of man and domestic animals on the African continent involving the area 15° North -29° South latitude (27). This Glossina (tsetse fly) infested area encompasses a land mass of approximately 4 million square miles. The disease of man, commonly referred to as African sleeping sickness, is present in endemic foci throughout this area making it hazardous or uninhabitable for humans. Insect vectors capable of transmitting the human disease, however, occur over a greater area increasing the potential for spread of this disease. Historically, large scale outbreaks occurred following the introduction of the trypanosomes into a previously unexposed population (5,15). During the period 1896-1906, over half a million people died in the Congo basin as the disease spread from the mouth of the river. During the epidemic of 1900-1910 in Uganda near Lake Victoria, an estimated 200,000 people were killed by the disease. More recently, in the 1930-1940 epidemic in East Africa, an estimated 11,500 human deaths were reported. Epidemics were followed by the establishment of persistent endemic foci. The current toll of more than 10,000 new cases reported annually, is considered to be an underestimate (3,4).

Animal trypanosomiasis is far more widely distributed. It may preclude or greatly reduce the production of domesticated animals for food or draft purposes. This deficit contributes to nutritional deficiencies and restricts social development (26).

There are 2 species of trypanosomes which cause human trypanosomiasis: Trypanosoma rhodesiense and Trypanosoma gambiense. Differentiation is based on the clinical syndrome as the parasites are morphologically indistinguishable (12,17). The Rhodesian form is a more acute disease which usually terminates in 1-12 months if not treated. It is characterized by recurrent fever, headache, irritability, insomnia, anemia, local edema with rapid development of congestive heart failure and often neurologic disorders. Gambian trypanosomiasis generally follows a less acute clinical course but terminates fatally when the central nervous system (CNS) becomes affected. Clinical features include marked lymph node enlargement, drowsiness, headache and ataxia. In some patients onset of CNS disease is delayed 2-6 years after demonstration of parasitemia.

A limited number of relatively toxic drugs administrated by injection are available for treatment of the disease in man (1,19,20, 21,31). Instances of drug resistance have been reported for all drugs now in use (31). Treatment with Suramin (Bayer 205) or pentamidine is fairly effective for either the Rhodesian or the Gambian form before CNS involvement has occurred. However, because CNS penetration of trypanosomes may occur early, additional follow-up treatment with drugs able to cross the blood - brain barrier is recommended. To cross this barrier, the arsenically based melaminyl compounds are now being used. Hospitalization is required for therapy because of the often severe nervous irritation and other toxic effects associated with this class of drugs (20,31).

Control of the disease has been directed toward reduction of contact between flies and man and by chemoprophylaxis. Fly numbers have been reduced by bush clearing and insecticide application. Avoidance of endemic areas has been practiced, though population movements away from such areas may be responsible for early dissimination of the human reservoir (14).

Chemoprophylaxis has been widely used in West and Central Africa with pentamidine being the drug of choice. Because of its serum binding property, pentamidine prophylaxis for the Gambian type is effective for at least 6 months (23). Suramin also has prophalactic properties for approximately 3 months but because of its nephrotoxicity and lack of effectiveness for the Gambian form, its usage has been restricted. In any case, following the introduction of trypanosomiasis into a new area it continues in an endemic form irrespective of the control measures used (5,15).

The study of immunologic aspects of the disease which could possibly lead to a means of control have received increasing attention in recent years. Several workers have had limited success in immunizing animals using killed suspensions of trypanosomes (13,22) or extracts of trypanosomes as immunogens (2,28). Recent experiments have shown that immunity can be induced in rodents, cattle and monkeys against blood form trypanosomes by immunization with live parasites attenuated by ionizing radiation (8,29). Experimental work with T. congolense and T. brucei has not been as promising. Attempts to immunize cattle with irradiated parasites did not result in a sterile immunity but immunized animals mounted a humoral response and prepatent periods were extended (6,7). A major problem with the irradiated vaccine model is that immune animals are not protected against heterologous strain or variant challenge (30) and no information exists on whether these animals are protected from a natural tsetse fly challenge.

Our studies describe immunization trials with T. congolense in bovines in the laboratory. We also report on a field study, which has taken place over the last 8 years, in which we determined the antigenic similarities of T. rhodesiense occurring in an endemic area of western Kenya.

MATERIALS AND METHODS

Trypanosoma rhodesiense serodemes in Lambwe Valley

To determine the antigenic composition and number of serodemes active in an endemic area (Lambwe Valley), isolates of T. rhodesiense were collected from patients at the Homa Bay Hospital near Lake Victoria, western Kenya, by members of the Kenya Medical Department. Blood samples from the patients were then injected intraperitoneally into rats and transported to our laboratory in Nairobi for study. The trypanosome isolates were tested by neutralization (25) with two separate antisera. The antisera were developed by long term

infection of two bovines, one with an isolate collected in 1972 and the other with an isolate obtained in 1974. The duration of the infection in these animals was 227 and 279 days, respectively. To date, 45 T. rhodesiense isolates over 8-year period have been tested.

Acquired immunity to trypanosomiasis

Animals - Bovines used in these studies were a predominantly Hereford breed and obtained from the Veterinary Department at Kabete or from other trypanosomiasis free areas of Kenya. All animals were dipped or sprayed with an acaracide weekly and received periodic treatment with Ranizole (Merck Sharpe and Dohme, B.V., Haarlem - Netherlands) to limit helminthic infections. During periods of poor pasture supplemental food was provided.

Parasites - The Trans-Mara I strain of T. congolense was used in these experiments. It was first isolated from an infected bovine in the Trans-Mara area near the Kenya-Tanzania border in 1966 and has subsequently been maintained in stabilate form at -80° with occasional passage in bovines or rodents. White albino mice obtained from the Veterinary Laboratory rearing facility were infected with this stabilate. When parasitemias reached satisfactory levels the mice were anesthetized with ether and bled by cardiac puncture. Trypanosomes were counted in a hemocytometer and diluted with phosphate buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum.

Induction of immunity to blood form trypanosomes - Bovines were infected intravenously with $1x10^4$ trypanosomes per 500Lb body weight unless otherwise noted. When animals became weak, severely anemic, and were near death, treatment was initiated with Berenil (Farbwerke Hoechst, Frankfurt (M) Germany) at levels of 1.05g or 2.10g active ingredient per 660Lb of body weight. This procedure of infection and therapeutic cure was repeated until these animals were resistant to an intravenous challenge of $1x10^4$ trypanosomes per 500Lb body weight.

Tsetse fly challenge - Infected adult Glossina morsitans were used to induce metacyclic infection. The tsetse fly colony was established from shipments of puparia supplied by Dr. A.M. Jordan, Tsetse Research Laboratories, Bristol, England. Standard rearing procedures were utilized (16). Newly emerged (<24 hours post-emergence) flies were allowed to feed on a bovine infected with the Trans-Mara I strain of T. congolense. They were exposed to the donor animal each morning for 14 consecutive days and then allowed to feed for 15 consecutive days on each of two non-infected animals. Infection in flies was verified by dissecting proboscis, midguts and salivary glands from a random sample of flies. Infected flies were allowed to feed on cattle immune to syringe challenge with blood stage trypanosomes of T. congolense and control animals not previously exposed to T. congolense. Each animal received an average of 468 tsetse fly bites over a two day period.

Monitoring of infections - The temperatures, parasite levels and hematologic parameters of experimental animals were monitored. Blood for Giemsa stained thick and thin films were obtained from the tip of the tail six days per week and blood for routine hematology was obtained twice weekly from the jugular vein in disodium ethylenediamine tetraacetic acid (EDTA). The hematology performed included erythrocyte, leucocyte and thrombocyte counts as well as determination of packed cell volumes and hemoglobin concentrations. Parasitemias were estimated by counting the number of trypanosomes per 100 leucocytes on Giemsa stained thick blood films and relating these values to the total leucocyte count per mm^3.

Verification of Immune Status - When animals which "self-cured" or were repeatedly infected and cured with Berenil developed no detectable parasitemia or clinical evidence of disease after a blood induced challenge, they were considered immune. Partially immune refers to those animals which received a single infection and treatment. Blood was subinoculated from immune animals into mice as an adjunct to determining their immune status.

In conducting the research in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

RESULTS

Survey of antigenic specificity of T. rhodesiense - During the past eight years 45 isolates of *T. rhodesiense* were collected from patients and tested against two different antisera in an attempt to determine the extent of antigenic variation occurring in the Lambwe Valley, western Kenya, over a period of time. As can be seen in Table 1, antisera against two different isolates of T. rhodesiense reacted with 60 and 61 percent of the isolates, respectively. Only 9 isolates, distributed over the 8-year period, were not neutralized by either antiserum.

Immunization Experiments - As shown in Table 2, there is a marked age resistance to primary infection with T. congolense in bovines. Eighty-five percent of 13 animals less than 1 year of age and 11% of the animals between one and two years old survived and "self-cured" the initial infection. All animals two years or older required treatment to survive. No differences in survival times were found between male and female animals of the same age group.

Five bovines, 25 to 54 weeks after "self-curing" the initial infection, received a primary challenge. All five animals were immune and showed no detectable evidence of infection while the control animals became patent and required treatment within 9 weeks (Table 3). Older animals did not "self-cure" the infection and required treatment to survive. As seen in Table 4, nine animals over one year of age that

received an initial infection but required Berenil therapy, were challenged 28-128 weeks after treatment. Five of these animals "self-cured" and 4 required treatment but at times extended beyond that of the initial infection. "Self-cure" of a primary challenge seems to be dependent on a longer initial exposure to the parasite and rechallenge within a year of the last detectable parasitemia. However, there was detectable resistance even in the animals challenged over two years after the treatment of the initial infection. Five of the animals which had either "self-cured" or required treatment of the primary challenge were then given a secondary and subsequently a tertiary challenge. No detectable infections resulted in the experimental animals from either challenge while controls developed typical infections and all required treatment.

Twelve bovines, immune or partially immune to blood form trypanosomes, were subsequently challenged with T. congolense infected G. morsitans (metacyclic trypanosomes from tsetse flies). This fly-borne challenge was 8-9 months after the animal's last exposure to blood form trypanosomes. Eight control animals, not previously exposed to T. congolense, were similarly challenged. As seen in Table 5 and Fig. 1, 55% of the animals immune to blood stage trypanosomes were also completely resistant to metacyclic challenge. Though 45% became patent, they exhibited a significantly lengthened prepatent period and reduced parasitemia when compared to controls. These animals manifested no clinical evidence of disease and ultimately, all "self-cured". The partially immune animals which had received only a single exposure to blood trypanosomes, demonstrated a lengthened prepatent period and reduced parasitemia when compared to controls. They also "self-cured". Of the control group, 7 of 9 animals required treatment for survival while 2 "self-cured". The 2 that "self-cured" were 1-2 years old and reflected the age resistance to infection previously described.

DISCUSSION

Three basic problems must be resolved before immunization could be considered a method for control of trypanosomiasis. The first is concerned with the number and stability of serodemes persisting in an endemic area over a period of time. Gray has shown antigenic similarities in both T. brucei and T. gambiense isolates, respectively, collected from various geographic areas of Nigeria leading one to suspect that a limited number of serodemes were involved in each species (9,10). In our studies, the results of neutralization tests of T. rhodesiense isolates collected from individuals in Lambwe Valley, over an 8-year period, showed a marked similarity of antigenic composition. Both antisera used in the tests neutralized the same as well as different isolates which were presumably, all variants of the same serodeme. The 9 isolates which were not neutralized by either antiserum may belong to the same or to one or more different serodemes. These findings demonstrate the limited diversity and stable antigenic composition of the parasite population. This reduces the likelihood that erratic changes take place in the antigenic character of trypanosomes in a

given area and indicates that a successful vaccine could retain its protective capacity over a relatively long period of time.

The second problem deals with antigenic variation; a process utilized by the parasite to evade the hosts immune response (11,18,24). Immunization by "self-cure" or infection and treatment results in exposure of the host to a broad spectrum of antigenic variants. The protection induced by these procedures is apparently manifested by an anamnestic response to most of the antigenic variants of one serodeme. While these methods of immunization are not practical procedures for man, it indicates the important finding that a finite number of antigenic variants are produced and that mutation is probably not involved to a great degree in the process of antigenic variation. Furthermore, it appears that exposure of a host to a significant proportion of variants of a serodeme results in protection against the entire serodeme.

The third and perhaps most important problem in the development of a vaccine involves the question of whether or not animals immune to blood forms are protected against tsetse fly challenge. While animals have been immunized against antigenic variants by various methods, (2,6,7,8,13,28,29), they are resistant only to homologous challenge and their resistance to tsetse fly challenge has not been tested. In our experiments employing natural tsetse fly challenge of animals previously shown to be immune to blood forms, we found a marked degree of immunity. Most immunized animals were completely protected and showed no parasitemia or clinical evidence of trypanosomiasis. The immunized animals which became patent did so at a later time than controls and only a transient low level parasitemia developed. These transient parasitemias were not accompanied by clinical signs. All controls developed typical T. congolense infections characterized by progressive weight loss, anemia, leucopenia and thrombocytopenia.

In summary, we have demonstrated that the antigenic character of the parasite population of T. rhodesiense from an endemic area was composed of perhaps as few as one serodeme which was antigenically stable over an 8-year period. We also found that immunity can be induced to blood and tsetse fly (metacyclic) forms by exposure of experimental animals to a broad spectrum of antigenic variants of the same serodeme. The sterile immunity described in our studies was long lasting. We believe these findings enhance the likelihood of immunologic control of trypanosomiasis.

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TABLE 1

RESULTS OF NEUTRALIZATION TESTS OF ISOLATES OF *TRYPANOSOMA RHODESIENSE* COLLECTED BETWEEN 1970 AND 1978 IN LAMBWE VALLEY, KENYA

Antiserum	No. Isolates tested	No. Neutralized*	Percent
Anti LVH-1	45	27	60
Anti LVH-2	44	27	61

* 9 isolates (20%) were not neutralized by either antiserum.

TABLE 2

THE EFFECT OF AGE ON *TRYPANOSOMA CONGOLENSE* INFECTIONS IN CATTLE

Age (Years)	Number of Animals	Median Survival* Time (Weeks-Days)	Range (Weeks-Days)	No. Self Cures (%)
0.5-1	13	78-0	8-6 to 78-0	11 (85)
1-2	11	24-4	5-5 to 30-6	2 (11)
2-3	11	11-5	5-5 to 78-0	0 (0)
3-4	5	6-3	6-1 to 13-6	0 (0)
4-5	2	6-8	4-2 to 9-0	0 (0)
5-6	2	8-1	8-0 to 8-3	0 (0)

* Based on time to treatment or day of death.

TABLE 3

RESULTS OF PRIMARY CHALLENGE OF PREVIOUSLY INFECTED, SELF-CURED ANIMALS

Initial Infection						Interval[3] (Wks-Days)	Primary Challenge ($1x10^4$/500Lbs.)		
An. No.	Age (Yrs)	Sex[1]	Dose per 500Lbs.	P.P.[2] (Days)	Last Patent Parasitemia (Wks-Days)		Age (Yrs)	P.P.[2] (Days)	Result (Wks-Days)
1	0.5	M	$2.8x10^3$	8	54-4	25-0	2.0	Not Patent	No Detectable Infection
2	1.3	MC	$1.0x10^4$	5	61-1	31-6	3.0	"	"
3	0.3	F	$1.0x10^4$	5	56-2	36-5	2.0	"	"
4	0.5	M	$2.9x10^3$	6	30-5	48-5	2.0	"	"
5	1.4	MC	$1.0x10^4$	5	38-2	54-5	3.1	"	"
Average of 3 control animals for primary challenge							3.1	4.7	T (9-3)[4]

1. F - Female; MC - Male Castrated; M - Male.
2. Prepatent period.
3. Time between last patent parasitemia and challenge.
4. Treated (Time since challenge).

TABLE 4

RESULTS OF PRIMARY CHALLENGE OF PREVIOUSLY INFECTED AND TREATED CATTLE

Initial Infection						Interval[3] (Wks.-Days)	Primary Challenge ($1x10^4$/500Lbs.)		
An. No.	Age (Yrs)	Sex[1]	Dose per 500Lbs.	P.P.[2] (Days)	Time to Treatment (Wks.-Days)		Age (Yrs)	P.P. (Days)	Results (Wks.-Days)
6	1.0	F	$6.8x10^6$	3	7-0	28-6	1.7	14	S.C.(17-0)[4]
7	4.4	F	$1.0x10^4$	6	9-0	30-0	5.2	10	S.C.(11-5)
8	2.7	F	$1.0x10^4$	5	11-5	42-2	3.8	18	S.C.(15-5)
9	2.6	F	$1.0x10^4$	5	6-6	47-1	3.7	13	T.(36-6)[5]
10	1.9	F	$1.3x10^5$	5	28-0	71-4	3.9	14	S.C.(4-4)
11	1.6	MC	$8.4x10^3$	6	5-5	86-0	3.4	8	T.(21-3)
12	1.9	MC	$1.0x10^4$	6	5-5	86-0	3.7	6	T.(11-5)
13	2.3	MC	$1.9x10^4$	5	11-0	122-5	4.9	6	T.(27-0)
14	3.4	F	$1.3x10^4$	5	5-1	128-4	6.0	6	S.C.(29-2)
Average of 8 control animals for primary challenge							4.1	5.5	T.(9-4)

1. F - Female; MC - Male Castrated.
2. Prepatent Period.
3. Time between treatment and challenge.
4. S.C. - Self Cure (Time of last patent parasitemia after challenge).
5. T - Treated (Time since challenge)

TABLE 5

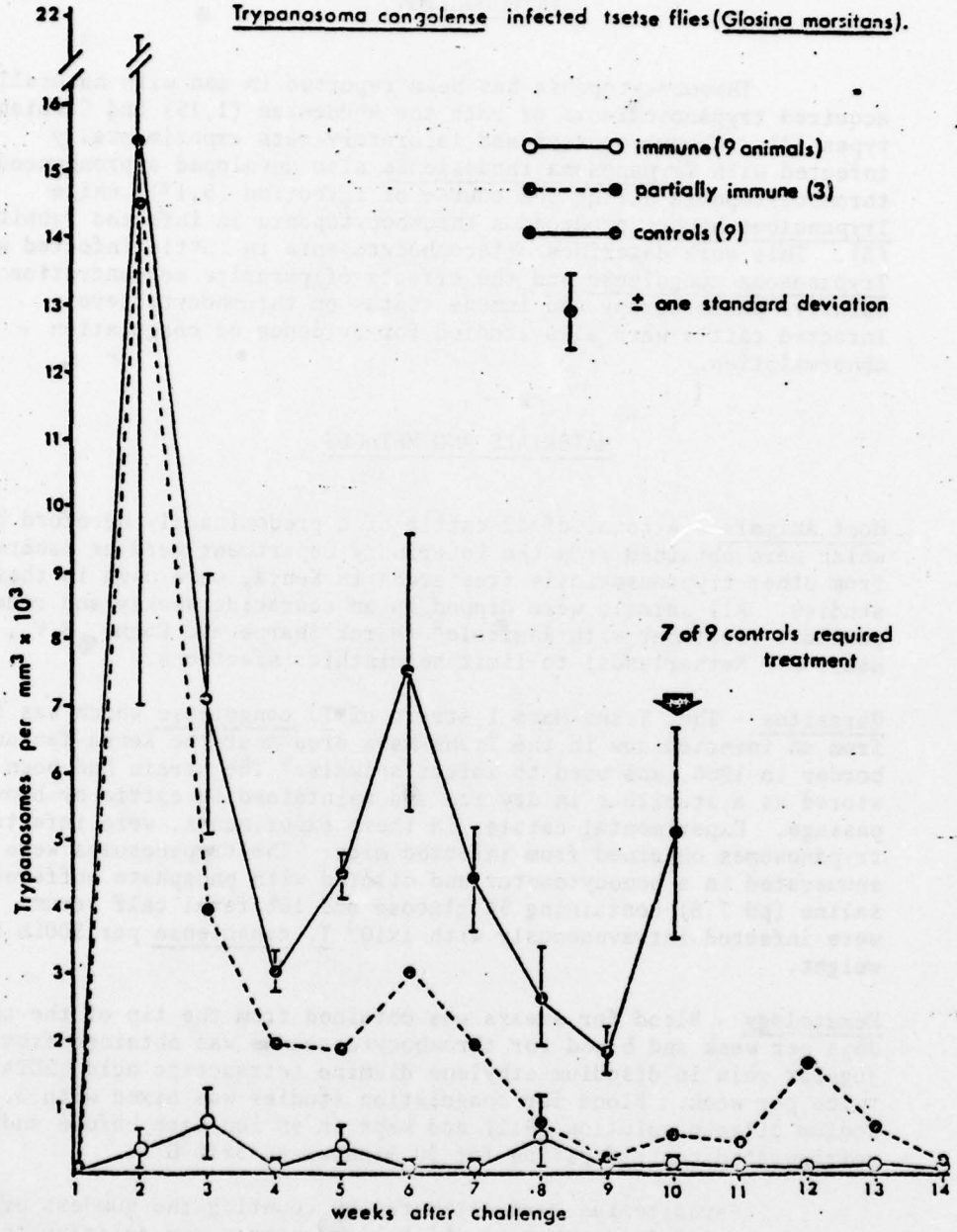
RESULTS OF CHALLENGE BY INFECTED TSETSE FLIES OF ANIMALS IMMUNIZED AGAINST BLOOD FORMS OF TRYPANOSOMA CONGOLENSE

Number of Animals	Group[1]	RESULTS			
		Median[2] P.P. (Days)	No Detectable infection	Self Cure	Treatment
9	Immune	180	5	4	0
3	Partially Immune	14.0	0	3	0
9	Control	10.0	0	2	7

1. Immune - resistant to last blood challenge.
   Partially Immune - one infection and treatment.
2. Prepatent period.

Figure 1

Average daily parasitemias of immune and control animals challenged by *Trypanosoma congolense* infected tsetse flies (*Glosina morsitans*).



TRYPANOSOMA CONGOLENSE: THROMBOCYTOPENIA IN EXPERIMENTALLY INFECTED CATTLE

INTRODUCTION

Thrombocytopenia has been reported in man with naturally acquired trypanosomiasis of both the Rhodesian (1,15) and Gambian types (7). Rhesus monkeys and laboratory rats experimentally infected with Trypanosoma rhodesiense also developed a pronounced thrombocytopenia during the course of infection (5,17), while Trypanosoma brucei produced a thrombocytopenia in infected rabbits (8). This work describes a thrombocytopenia in cattle infected with Trypanosoma congolense and the effects of parasite concentration, curative chemotherapy and immune status on thrombocyte levels. Infected cattle were also studied for evidence of coagulation abnormalities.

MATERIALS AND METHODS

Host Animals - A total of 32 cattle of a predominantly Hereford breed which were obtained from the Veterinary Department herd at Kabete, or from other trypanosomiasis free areas in Kenya, were used in these studies. All animals were dipped in an acaracide weekly and received periodic treatment with Ranizole^{R} (Merck Sharpe and Dohme, B.V., Haarlem - Netherlands) to limit helminthic infections.

Parasites - The Trans-Mara I strain of T. congolense which was isolated from an infected cow in the Trans-Mara area near the Kenya-Tanzania border in 1966, was used to infect animals. The strain had been stored as a stabilate in dry ice and maintained in cattle by blood passage. Experimental cattle, in these experiments, were infected with trypanosomes obtained from infected mice. The trypanosomes were enumerated in a hemocytometer and diluted with phosphate buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum. Cattle were infected intraveneously with $1x10^4$ T. congolense per 500Lb body weight.

Hematology - Blood for smears was obtained from the tip of the tail six days per week and blood for thrombocyte counts was obtained from the jugular vein in disodium ethylene diamine tetraacetic acid (EDTA) usually twice per week. Blood for coagulation studies was mixed with 3.8% sodium citrate solution (9:1) and kept in an ice bath before and after refrigerated centrifugation for 20 minutes at $3x10^3$G's.

Parasitemias were estimated by counting the numbers of trypanosomes per 100 leucocytes on thick blood smears and relating these values to the total leucocyte counts per mm^3. Thrombocyte numbers were determined by phase microscopy (4). The method of Quick (13) was used for prothrombin determinations and activated partial thromboplastin times were done using Celite^{R} (General Diagnostics, Morris

Plains, N.J.) as the particulate activator (12). The plasma protamine paracoagulation test described by Seaman (18) was modified by using 0.6cc of test plasma instead of 1cc. Fibrinogen levels were determined by a gravimetric method (11). Duplicate tests were done on all plasma samples and the averages recorded.

Chemotherapy - Curative chemotherapy was initiated with Berenil^{R} (Farbwerke Hoeschst, Frankfurt (M) Germany) at a level of 1.05g active ingredient per 660Lb of body weight. Animals which had been repeatedly infected and cured, and which showed complete or partial immunity upon homologous rechallenge are referred to as being immune or partially immune respectively.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals, as promulgated by the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

RESULTS

Normal thrombocyte levels

A series of counts were made from normal adult and immature cattle during a two month period in order to establish normal thrombocyte values for animals at this laboratory (Table 1). These have been compared with values from animals experimentally infected with T. congolense in Figures 2-5.

Thrombocyte levels in infected animals

The first experiment compared the levels of thrombocytes in 14 adult infected and 6 control animals (Fig. 1). When infected animals were judged to be near death they were treated and data collected thereafter are presented in Figure 4. A thrombocytopenia developed when parasites first appeared and was most severe during the early weeks of the infection when the level of parasitemia was greatest. During this period, spontaneous clumping of thrombocytes in the counting chamber was most prominent. As the level of trypanosomes in the peripheral circulation slowly diminished, the number of platelets gradually increased, until levels in surviving animals had returned to normal. The inverse relationship between parasitemia and thrombocytopenia was more evident in the data for the individual animals since animals with the highest levels of trypanosomes in the peripheral blood had the most pronounced thrombocytopenia. Fig. 2 shows the course of disease in an animal with an acute infection. Thrombocyte levels decreased to $5x10^{4}mm^{3}$ soon after infection and had recovered only slightly by day 48 when the animal was treated. At the time of treatment the animal was prostrate and undergoing a convulsive episode. Its blood appeared to clot normally, but blood tinged mucous was found in the stool. One other acutely infected animal in the study experie-

nced the same signs but also had periorbital edema and petechial hemorrhages in the eyelids, sclera and nostrils.

In a second experiment, 6 T. congolense infected calves also developed a thrombocytopenia when compared with 6 controls. Since normal thrombocyte values in calves were higher than those of adults (Table 1), the average levels of thrombocytes in infected calves were also higher than those of infected adults. Counts below $1x10^5$ per mm^3 were unusual in individual calves while averages for the group usually did not go below $2x10^5$ thrombocytes per mm^3. No detectable differences in thrombocyte levels were observed between sexes in control or infected animals.

Animals in a chronic state of infection had fluctuating patterns of thrombocytopenia which were inversely related to parasite levels (Fig. 3a & b). During or shortly after a parasite recrudescense there were fewer thrombocytes, while during periods of remission, values usually returned to within normal limits or higher.

Effects of treatment

Data for 7 animals treated during the course of T. congolense infection are presented in Fig. 4. Thrombocyte levels and parasitemia are compared 4 weeks before and after treatment. While low platelet levels and parasitemia persisted for the 4 weeks before treatment, the post treatment period was characterized by a lack of parasitemia and a rapidly rising thrombocyte level which reached greater than normal limits and then gradually subsided. Berenil^R treatment had no effect on platelet levels in 3 uninfected control animals studied during the same period. Animals immunized by repeated infection and cure did not develop parasitemia or thrombocytopenia while partially immune animals developed a pattern of parasitemia and thrombocytopenia similar to that seen in chronic infections.

Coagulation tests

Plasma from six infected and three control animals in experiment number 1 was tested weekly to determine prothrombin times and partial thromboplastin times during the first fourteen weeks of infection. Tests were done on 5 consecutive days during week eight and twice per week during weeks 9 and 10. No significant differences were found between prothrombin times of infected and control animals during this period. Partial thromboplastin times of the two groups were also similar for the first 6 weeks after infection. After the sixth week, however, values from plasma collected from infected animals showed a consistently prolonged PTT of from 7-18 seconds over those of control (Fig. 5).

In Fig. 6, fibrinogen levels from 5 infected animals are plotted against 18 values from 3 normal animals. Although not severely lowered, levels in infected animals were consistently below those of controls.

Plasma protamine paracoagulation tests were conducted from week 8 through 14 after infection and all 5 infected animals whose plasma was tested were positive on at least one occasion during this period. Plasma from control animals was never positive in the test (Table 2).

DISCUSSION

Our studies have shown that thrombocytopenia occurs in cattle infected with T. congolense. The inverse relationship found between parasite and thrombocyte levels indicates an effect either by the parasite or its products on the megakaryocytes or on the thrombocyte per se. Bone marrow aspirates appeared to have at least a normal number of megakaryocytes (20) therefore, a suppression of production would presumably involve a slow down or halt in budding or release of thrombocytes from the megakaryocyte. An interpretation of our data point more to thrombocyte destruction accompanied by an increased thrombocyte synthesis. The rapid development of a thrombocytosis in previously thrombocytopenic animals after curative therapy, strongly suggests that a high level of production and turnover had been in process throughout the parasitemic phase. The rapidly rising thrombocyte levels found during remissions of parasitemia again indicates an increased level of production. Survival times of Cr^{51}-labelled thrombocytes have also been shown to be greatly reduced in T. congolense (Trans-Mara I strain) infected cattle (Preston, J.M., Kovatch, R.M., and Wellde, B.T., unpublished data).

Several mechanisms of destruction, as the process responsible for the thrombocytopenia, can be suggested. Antigen-antibody complexes have been recognized as mediators of thrombocyte destruction (19). Immune adherence was first described using an in vitro trypanosome thrombocyte system (14). This reaction of antibody, complement, and trypanosomes attaching to a complement receptor site on the thrombocyte surface could lead to their increased sequestration and destruction. Receptor sites on the thrombocyte with high affinity for trypanosomes could initiate thrombocyte-trypanosome association without the presence of antibody or complement. In a comprehensive series of experiments using T. rhodesiense infected rats, however, thrombocyte aggregation occurred when trypanosomes or supernatants from lysed trypanosomes were added to thrombocyte suspensions. This reaction was not affected by inhibitors of adenosine diphosphate, kinins or components of complement, but was heat labile (5).

The relationship between immunopathologic types of destruction and that caused by disseminated intravascular coagulation (DIC) is not clearly defined since DIC may also be initiated by antigen-antibody complexes (2,10,13). Evidence for a coagulopathy has been found in humans with T. rhodesiense infection (1,16), and dogs infected with the Trans-Mara I strain of T. congolense (Johnson, A.J., Dept. of Pathology, Walter Reed Army Institute of Research, Wash., D.C. 20012, personal communication). Elevated levels of fibrinogen degradation products have been shown to occur in T. brucei-infected rabbits (13).

The increased release of thromboplastic substances associated with persistent platelet destruction could activate coagulation mechanisms. Our findings of extended partial thromboplastin times between 6 and 14 weeks after infection along with lowered fibrinogen levels and positive plasma paracoagulation tests indicates that this occurs. The coagulopathy in bovines infected with the Trans-Mara I strain, however, is not severe and we observed no marked tendency for hemorrhages to occur. Petechial hemorrhages have been seen occasionally, however, in the sclera, nostrils and visceral organs of acutely infected cattle. Since the integrity of the capillary endothelium depends on an adequate level of thrombocytes (9), a more virulent strain of parasite, by producing higher parasitemia, might reduce the thrombocyte levels in cattle to a degree associated with more obvious and severe bleeding. In man, thrombocyte levels generally have to fall below $2x10^4$ per mm^3 before spontaneous hemorrhage is likely to occur and only 8% of patients having platelet counts between levels of $5x10^4$ to $1x10^5$ developed hemorrhages (6).

While the etiology of the thrombocytopenia is not certain, there may be a primary interaction between the thrombocyte and the trypanosome or its products which leads to thrombocyte destruction. As previously suggested (5) increased thromboplastic activity may secondarily activate coagulation mechanisms which in different species may lead to consumptive coagulopathies with varying degrees of severity.

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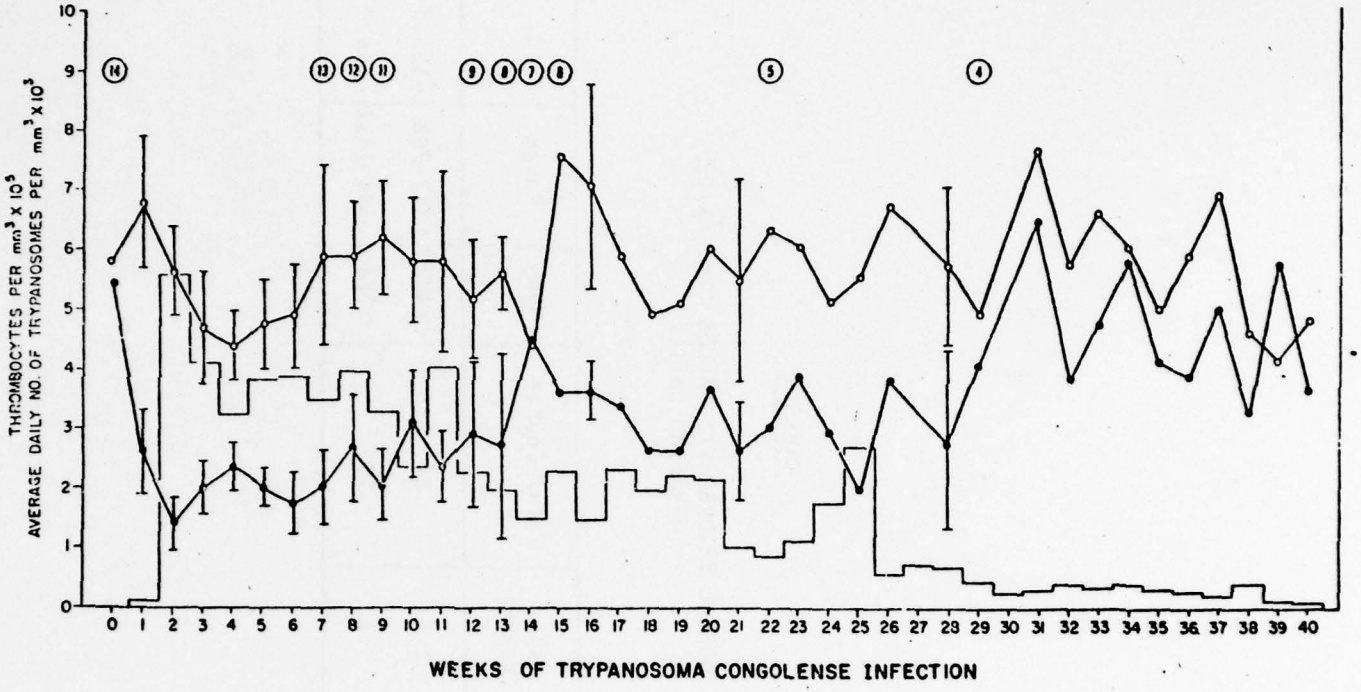


FIG. 1. Average thrombocyte and parasitemia levels in Trypanosoma congolense-infected and control cattle. ●—●, infected ± 2S.E.; ○—○, control ± 2S.E.; ⊏⊐, parasitemia; O, number infected animals remaining. Two standard errors have not been plotted for data points where infected and control values (± 2S.E.) overlap.

TABLE 1

NORMAL THROMBOCYTE VALUES ($1x10^3$) OF
HEREFORD CATTLE

No. Animals	Age (Years)	Total Counts	Mean	Range	S.D.	S.E.
14	1-4	84	492	212-798	±155.3	±16.9
10	1	70	784	344-1452	±248.0	±29.6

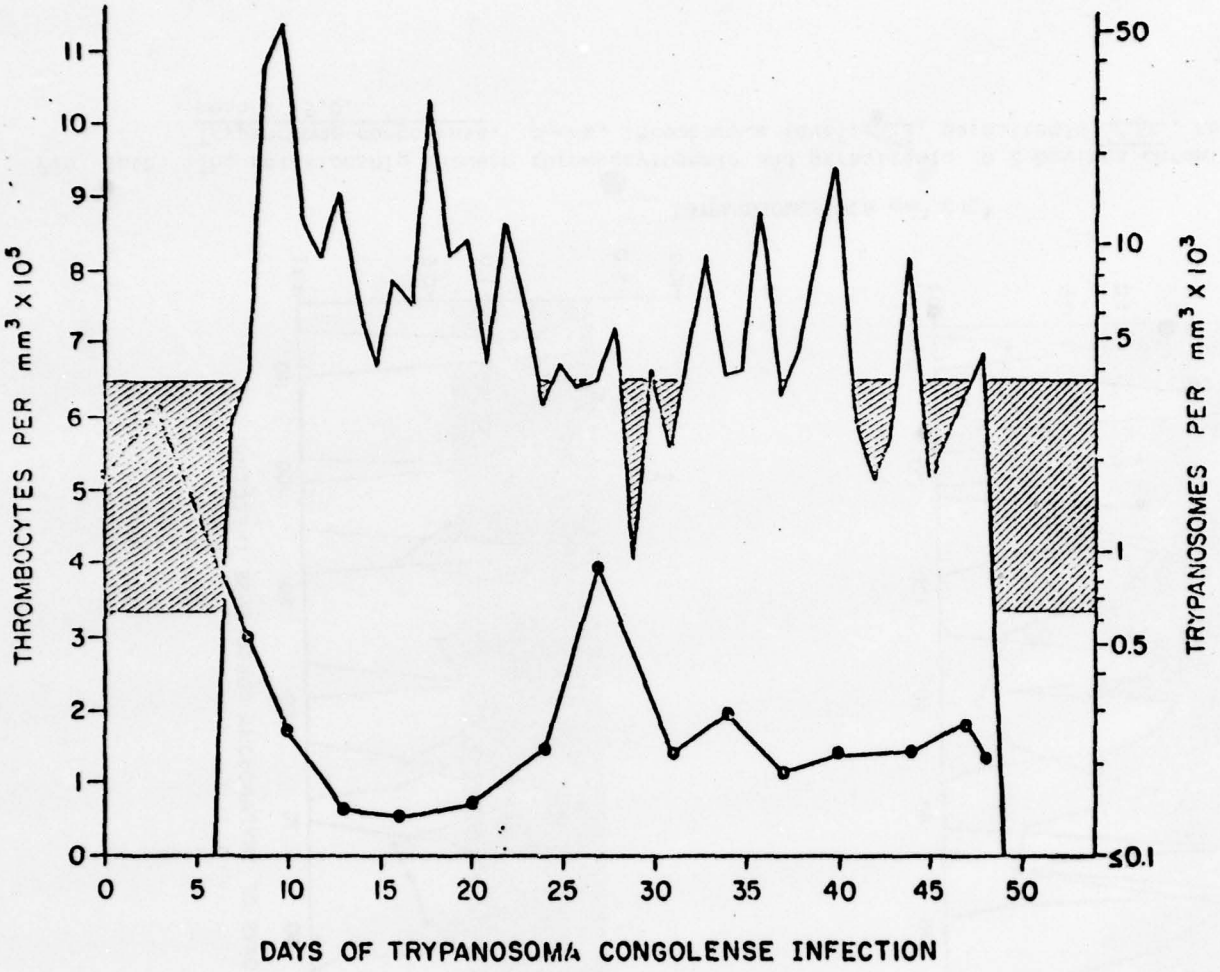


FIG. 2. Thrombocyte and parasitemia levels in a bovine acutely infected with Trypanosoma congolense. ●—●, thrombocyte level; ▭, parasitemia; ▨, normal thrombocyte mean ± 1S.D.

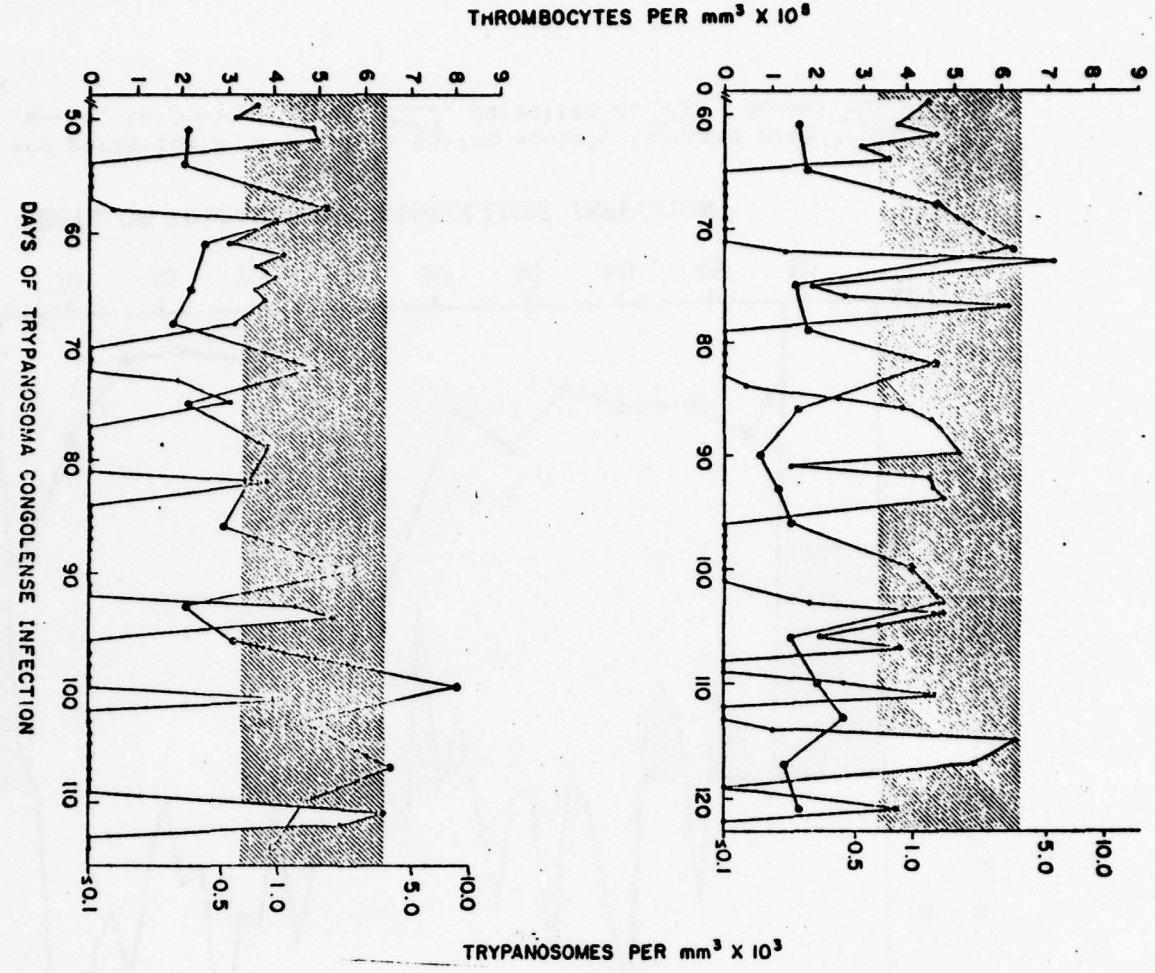


FIG. 3a&b. The relationship between thrombocytopenia and parasitemia in 2 bovines chronically infected wit Trypanosoma congolense. ○—●, thrombocyte level; ▭, parasitemia, ▭, normal thrombocyte mean ± 1S.D.

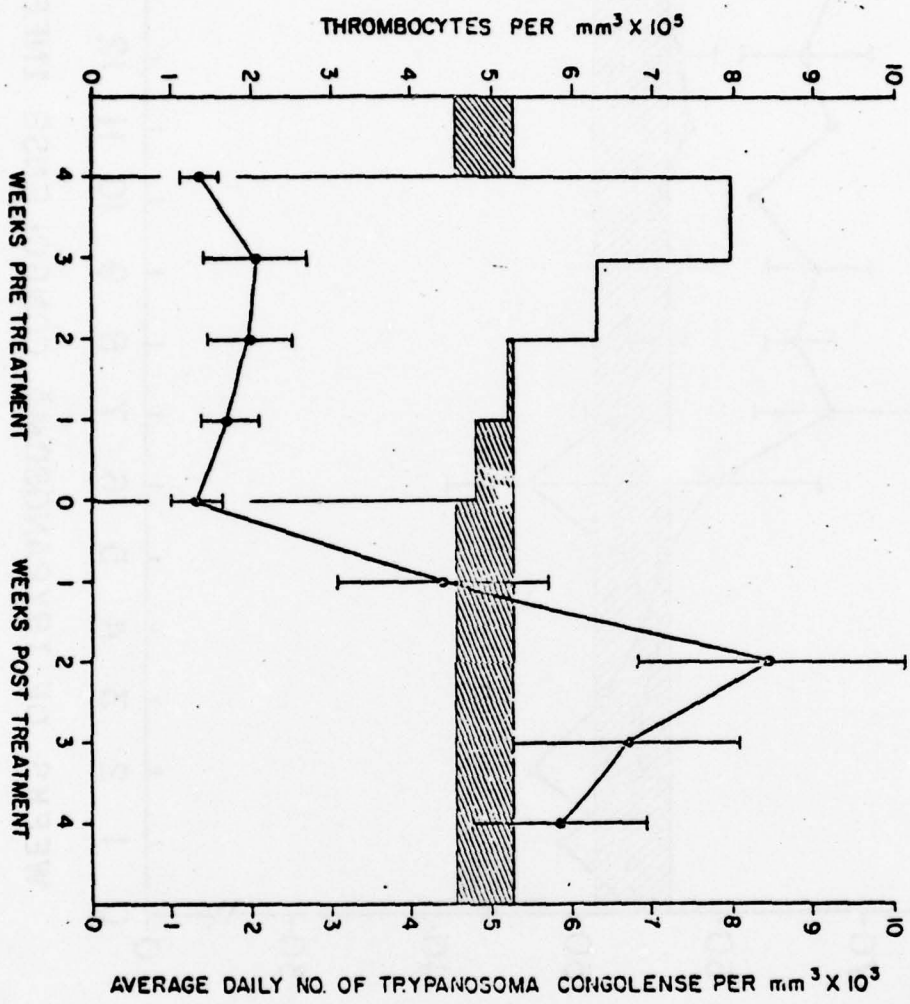


FIG. 4. The effect of curative chemotherapy on average thrombocyte and Trypanosoma congolense levels in cattle. ⟷, thrombocyte level ± 2S.E.; ⌐¬, parasitemia; ▨, normal thrombocyte level ± 2S.E.

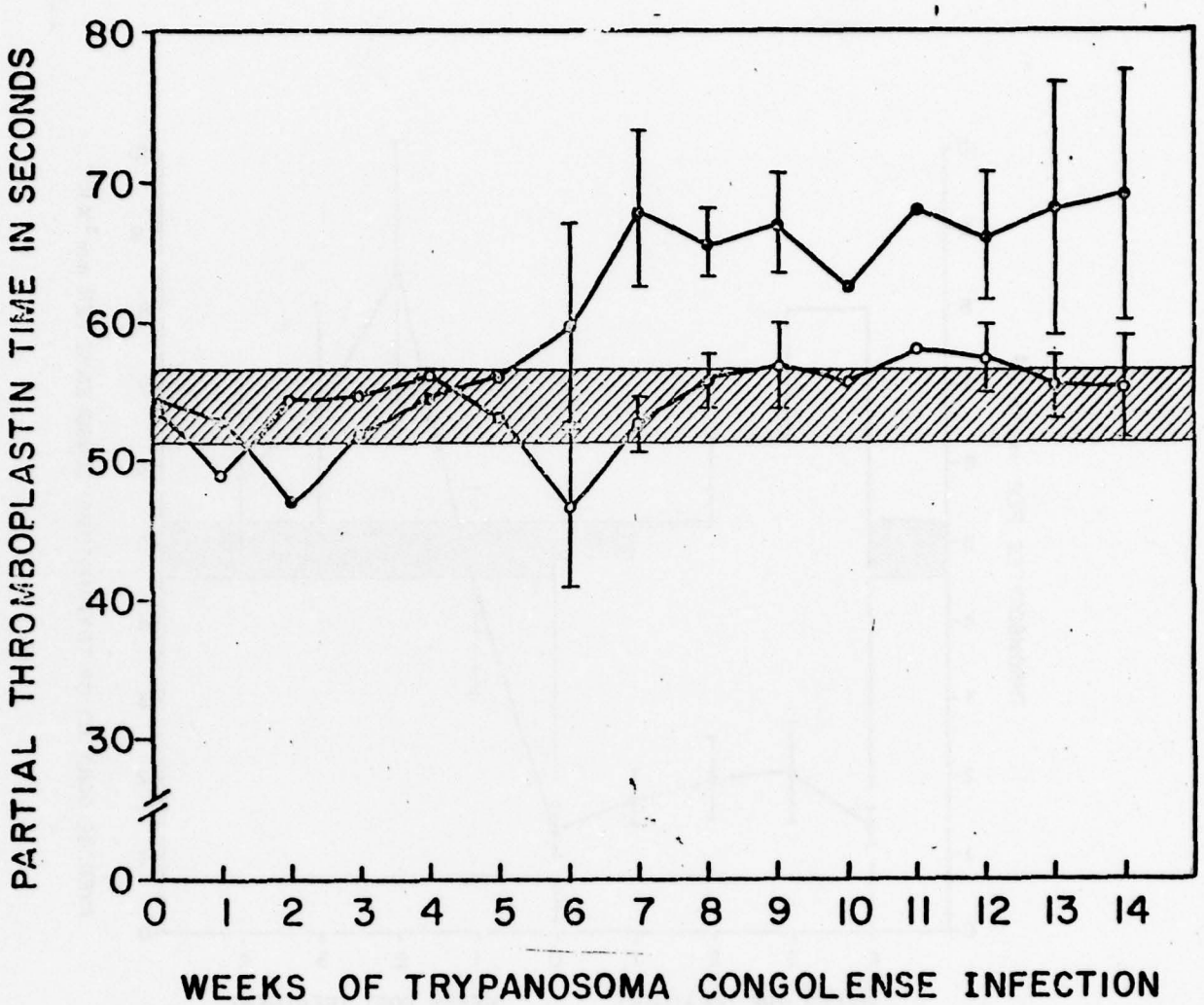


FIG. 5. Partial thromboplastin times of Trypanosoma congolense infected and control cattle. ●—●, infected ± 2S.E.; o—o, control ± 2S.E.; ZZZZ, normal partial thromboplastin time ± 2S.E. (27 determinations from 9 animals). Two standard errors have not been plotted for data points where infected and control values (± 2S.E.) overlap.

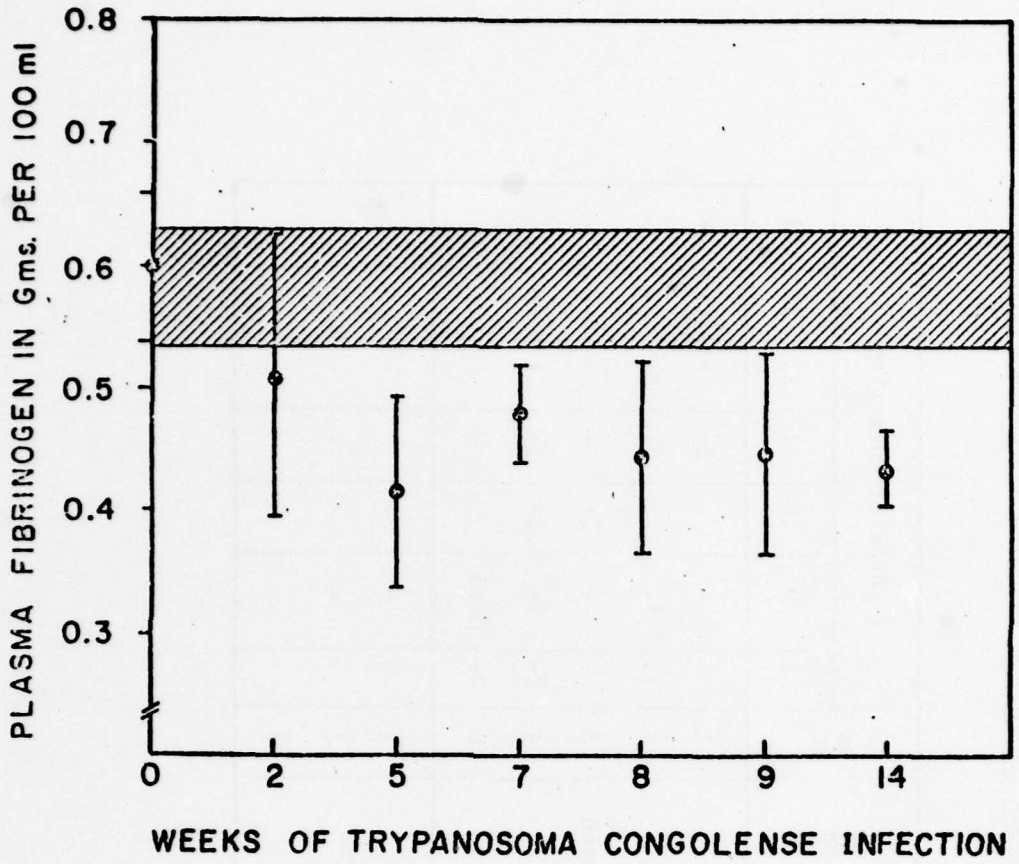


FIG. 6. Fibrinogen levels in Trypanosoma congolense infected and control cattle. ●—●, Infected ± 2S.E., ////, normal fibrinogen level ± 2S.E. (18 determinations from 3 animals).

TABLE 2

RESULTS OF PLASMA PROTAMINE PARACOAGULATION TESTS ON *TRYPANOSOMA CONGOLENSE* INFECTED AND CONTROL ANIMALS

Group	An. No.	Week of Infection						
		8	9	10	11	12	13	14
Infected	1	-	-	-	2+	4+	-	2+
	2	1+	1+	-	2+	3+	2+	2+
	3	-	-	2+	-	-	-	-
	4	2+	2+	3+	3+	2+	1+	2+
	5	4+	Dead					
Control	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-

TRYPANOSOMA CONGOLENSE: ERYTHROCYTE SURVIVAL IN INFECTED CALVES

INTRODUCTION

Anemia is a characteristic of many salivarian trypanosome infections and varies in intensity with the species of trypanosome and host. Although previous studies have shown that cattle infected with Trypanosoma congolense rapidly develop a severe anemia (1,8,10), the mechanisms which produce the anemia have yet to be adequately described. While some investigators have suggested that a hemolytic process is involved, others have found evidence of ineffective erythropoiesis and hemodilution (1,6,8). Working with Hereford cattle infected with T. congolense, Wellde et al (10) described an anemia which appeared to be hemolytic during the early stages of infection when parasitemias were at high levels. In animals which survived this initial phase, however, the anemia was more typical of that caused by decreased or impaired erythropoiesis.

Since these observations were based on conventional hematological procedures, a series of experiments using radioisotopic tracers was designed to study the kinetics of the anemia in the Hereford model. In the studies reported here, autologous erythrocytes labelled with ^{51}Cr-sodium chromate were transfused into control and infected animals at intervals throughout infection in an attempt to determine the relative importance of hemolysis and hemodilution in the development of anemia caused by T. congolense in cattle.

MATERIALS AND METHODS

Experimental design

Four male Hereford calves were used in each of 2 separate experiments. The calves, which averaged 110kg in weight and ranged from 6-8 months of age at the beginning of the experiments, were obtained from a trypanosomiasis free area of Kenya. The calves were fitted with fecal collection bags and kept indoors in metabolism cages throughout the period of study. Feed consisted of hay and water ad libitum supplemented with a commercial calf rearing concentrate. All calves in the study were treated with Phenamidine (May and Baker, Dagenham, England) at a rate of 1.5ml/45kg and Terramycin (Pfizer International, New York) at 10mg/45kg, 4 weeks before experimentation. The calves, which were fascioliasis free, were also given 2 treatments of Ranizole (Merck Sharpe and Dohme, Hoddesdon, England), to remove any gastrointestinal worms. The calves were injected with autologous ^{51}Cr-labelled erythrocytes one week before and 4,10,18 and 28 weeks after infection with T. congolense. The animals were weighed at the time of each injection.

Parasitological and clinical techniques

The Trans-Mara I strain of T. congolense was used in these studies and its history has been previously described (10). Trypanosomes obtained from infected mice were enumerated in a hemocytometer and diluted with phosphate buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum. Animals were injected intravenously with

$1x10^4$ T. congolense per 240kg body weight. Parasitemias were estimated by counting the numbers of trypanosomes per 100 leucocytes on thick blood films and relating these values to the total leucocyte count. Blood for smears was obtained each day from the tip of the tail. Rectal temperatures and clinical observations were also made daily.

Hematological methods

Packed cell volumes (PCV) were measured by the microhematocrit method. Erythrocyte and leucocyte counts were done using an electronic counter (Coulter Electronics, Harpenden, England) and hemoglobin levels were determined by the cyanmethemoglobin method (9).

Radioisotopic techniques

Erythrocytes were labelled with ^{51}Cr as previously described (3). Approximately 15ml of heparinized erythrocytes were incubated with 2.5ml $Ci^{51}Cr$ (high specific activity) as sodium chromate in physiological saline for 30 minutes at 37°C. After washing three times in physiological saline, the cells were resuspended in their own plasma prior to injection. A measured weight of autologous labelled erythrocytes was injected through a jugular catheter. Blood samples were collected into tubes containing disodium ethylenediaminetetraacetic acid (EDTA) from the contralateral vein 15 minutes after each injection and daily thereafter. Aliquots of blood (1.0ml) were diluted to 15ml with 0.01 N NaOH and the radioactivity measured on an automatic gamma scintillation counter (Packard Instruments, Illinois). The total daily output of feces and urine was also measured and aliquots of each taken for assessment of radioactivity.

Calculations

The blood volumes were calculated from the total radioactivity injected and the radioactivity of the 15 minute post-injection blood sample, using the isotope dilution principle. The circulating erythrocyte volume was derived from the blood volume and the venous hematocrit and the plasma volume calculated as the difference between the two values. All volumes were expressed as ml/kg body weight.

The apparent erythrocyte half-life ^{51}Cr ($RBC^1/_2$) was obtained from the regression equation of a semi-logarithymic plot of erythrocyte activity against time. Daily fecal and urinary erythrocyte "clearance" values were calculated by dividing the total daily excreted activity of each by the activity per ml of erythrocytes on the previous day. Mean values quoted in the test are given ± 2 Standard errors (SE).

RESULTS

Clinical and parasitological findings

The prepatent periods ranged from 5-7 days. Levels of parasitemia (Fig. 1) rose rapidly and remained at high levels for the first 6 weeks. From the sixth to the thirteenth week levels gradually decreased and parasitemias remained at relatively low levels for the remainder of the experiment. Fevers (105°F) were associated with the first peak of parasitemia and were observed intermittently thereafter.

Transitory inappetence and reduced weight gain characterized the first month of infection. The infected calves then became progressively weaker and lost weight. Over the 28-week period infected calves lost an average an average of 3.5kg while controls gained 36.2kg. All infected and control calves survived the experimental period.

Hematological manifestations

With the onset of patency, the PCV of the parasitised calves progressively decreased to a value of 21.1±2.5% as compared with 33.1±2.1% for the controls at 10 weeks after infection (Fig. 2). After this time, there was a gradual increase of PCV's until the termination of the study at 28 weeks post infection when the infected and control calves had values of 27.5±1.0% and 34.0±1.7% respectively.

In general, the alterations in erythrocyte count and hemoglobin concentrations followed a pattern similar to that of the PCV. However, there was a trend towards an increased mean corpuscular volume (MCV) and an increased mean corpuscular hemoglobin (MCH) at 10 weeks post infection when the PCV's were most severely lowered.

Blood, plasma and circulating erythrocyte volumes

The mean values of the blood, plasma and circulating erythrocyte volumes determined throughout the course of the experiment are shown in Fig. 3. During the initial 10 weeks after infection, the circulating erythrocyte volume fell from a mean of 19.5±1.8ml/kg to 10.3±1.0ml/kg representing a reduction of 47.2%. In contrast, the plasma volume of the same animals increased from 30.3±3.4ml/kg to 39.4±3.3ml/kg during this period and resulted in no significant alteration to the total blood volume. Between the 10th and 28th weeks the circulating erythrocyte volume of the parasitised animals increased progressively to 14.3±1.2ml/kg while the blood volume remained unchanged.

^{51}Cr-labelled erythrocyte turnover studies

The apparent erythrocyte survival times (^{51}Cr RBC $T^{1}/2$) for control and infected animals are shown in Fig. 4. In parallel with the declining PCV the apparent survival times of erythrocytes in the infected animals were reduced as early as the first two weeks after infection and further reductions were recorded during the following 12 weeks. The greatest differences in apparent survival times between infected and control animals was found during the fourth to the sixth weeks of infection when values for controls averaged 321±30 hours as compared with 128±46 hours for infected animals. After this period the ^{51}Cr RBC $T^{1}/2$ values increased to a mean of 243±43 hours in infected animals compared to 304±11 hours in the controls at 28 weeks after infection.

Estimates of the daily gastro-intestinal erythrocyte loss by the control and infected calves are shown in Table 1. The results measured over a total period of 12 weeks after infection show no significant differences between the two groups.

The amount of radioactive label excreted in the urine of both groups of animals is shown in Table 1. The values are expressed as erythrocyte "equivalents" for the period of 2-4 weeks after injection of ^{51}Cr-labelled cells. The 39.5-47.5ml daily urinary erythrocyte excretion of controls is due to continued elution of the label from the erythrocytes. It can be seen, however, that the loss of erythrocyte equivalents in the urine of the infected animals was much greater than that of the controls.

DISCUSSION

Our results show clearly that the apparent survival time of ^{51}Cr-labelled erythrocytes was greatly reduced in T. congolense infected calves when compared to that of controls. This finding, coupled with that of excess urinary excretion of the isotope by infected animals, indicates that the anemia was hemolytic in nature. While ^{51}Cr-labelled erythrocytes have proved useful for determining durations of erythrocyte survival in man and experimental animals, chromium elution from the cells complicates the calculation of the true survival time. In man ^{51}Cr-labelled erythrocytes have apparent survival times of 28 to 32 days as compared with life spans of 110-120 days as determined by cohort isotopic iron labelling (2). Higher elution rates of ^{51}Cr are associated with erythrocytes of domestic animals and in our studies an average apparent survival span of 331 hours was found for 26 determinations on 4 animals. Other investigators, using a ^{51}Cr label, have reported apparent survival times of bovine erythrocytes ranging from 216 to 430 hours (6,7).

The apparent erythrocyte survival time in our studies, was shortest during the early period of infection when the parasitemia was at the highest levels. As the parasitemia gradually subsided, the apparent survival times and packed cell volumes increased. In earlier studies, however, the inverse relationship between packed cell volume and parasitemia was not evident in late stages of the disease (10). Animals in those studies remained severely anemic even though trypanosome were found only intermittently and at low levels. This suggests that a second mechanism, aside from increased destruction, perpetuated the anemia in some animals. Whether age or nutritional status play a role in these differing observations remains to be determined.

Results presented by others are in agreement with our findings of shortened apparent erythrocyte survival times in T. congolense infected animals. In a brief experiment, Mamo and Holmes (6) studied 3 T. congolense infected bovines and 3 controls over a 12-day period and found a significant reduction in the life span of ^{51}Cr-labelled erythrocytes in the infected animals. A shortened life span was also found for syngeneic ^{51}Cr-labelled erythrocytes in T. congolense infected mice and was accompanied by a progressive increase in osmotic fragility of erythrocytes (4).

The mechanisms responsible for the decreased survival span and increased destruction of erythrocytes in infected animals are not known. It has been reported that immunoglobulins, eluted from erythrocytes

obtained from infected animals, reacted with T. congolense antigens in a series of serological tests (5). This evidence suggests that passive sensitization of erythrocytes by antibody or more likely, immune complexes, rendered the erythrocyte more susceptable to phagocytosis. While it appears that extrinsic agents act upon normal erythrocytes to produce the anemia, the possibility that at some stage of the infection an intrinsically defective erythrocyte may be produced cannot be dismissed as yet.

In our studies the packed cell volume was a satisfactory indicator of the degree of anemia, since no marked fluctuations occurred in total blood volumes. Other investigators have claimed that hemodilution plays an important role in the decreased packed cell volume and interpretation of anemia (1,6,8). These findings were not confirmed by blood volume determinations using ^{51}Cr-labelled erythrocytes in our experiments.

While the anemia accompanying T. congolense infections in cattle has been shown to have an important hemolytic component we are pursuing ferrokinetic studies in animals, at various stages of infection, to determine whether or not other factors play a role.

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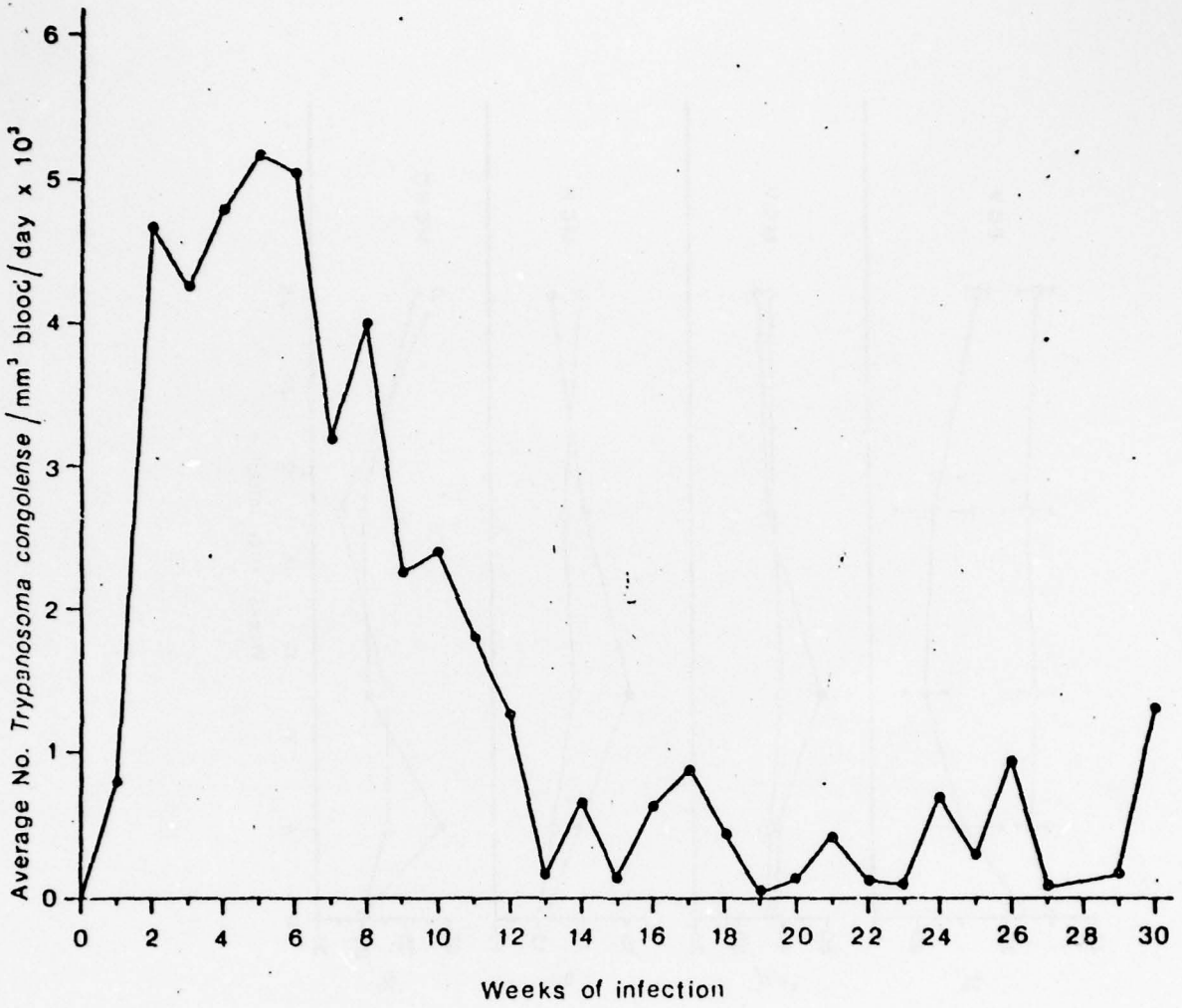


FIG. 1. Average parasitemias of Trypanosoma congolense infected calves.

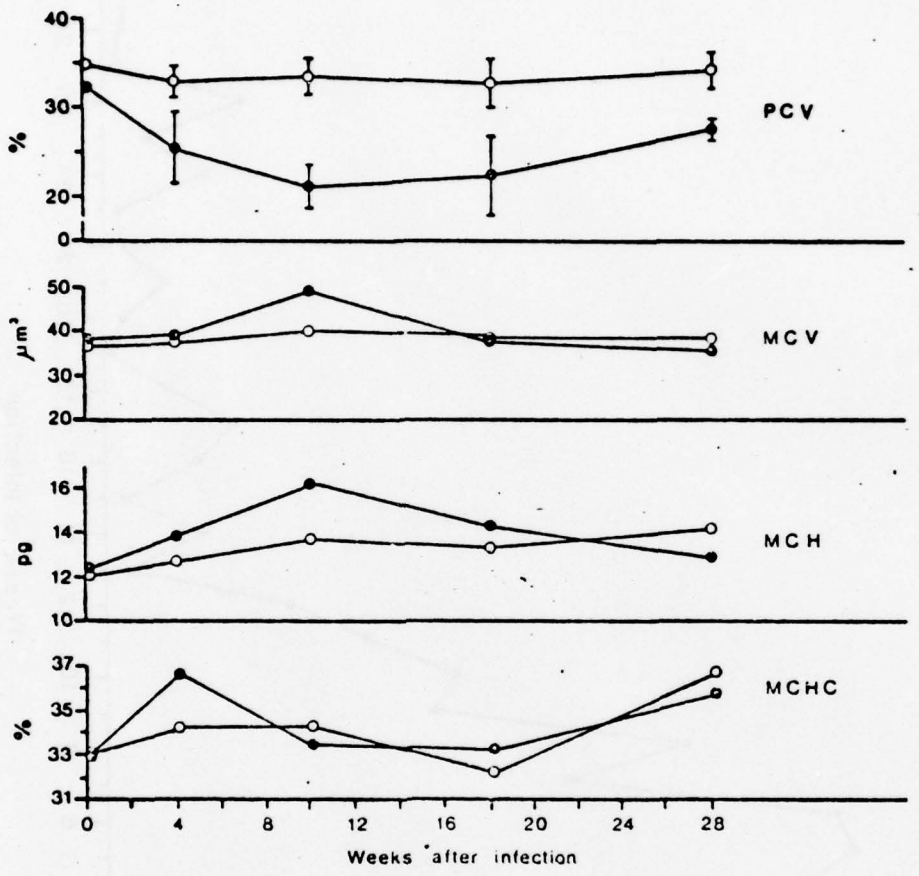


FIG. 2. Hematologic parameters of Trypanosoma congolense infected calves. o, control; •, experimental. ± 2S.E. are not plotted where experimental and control values overlap.

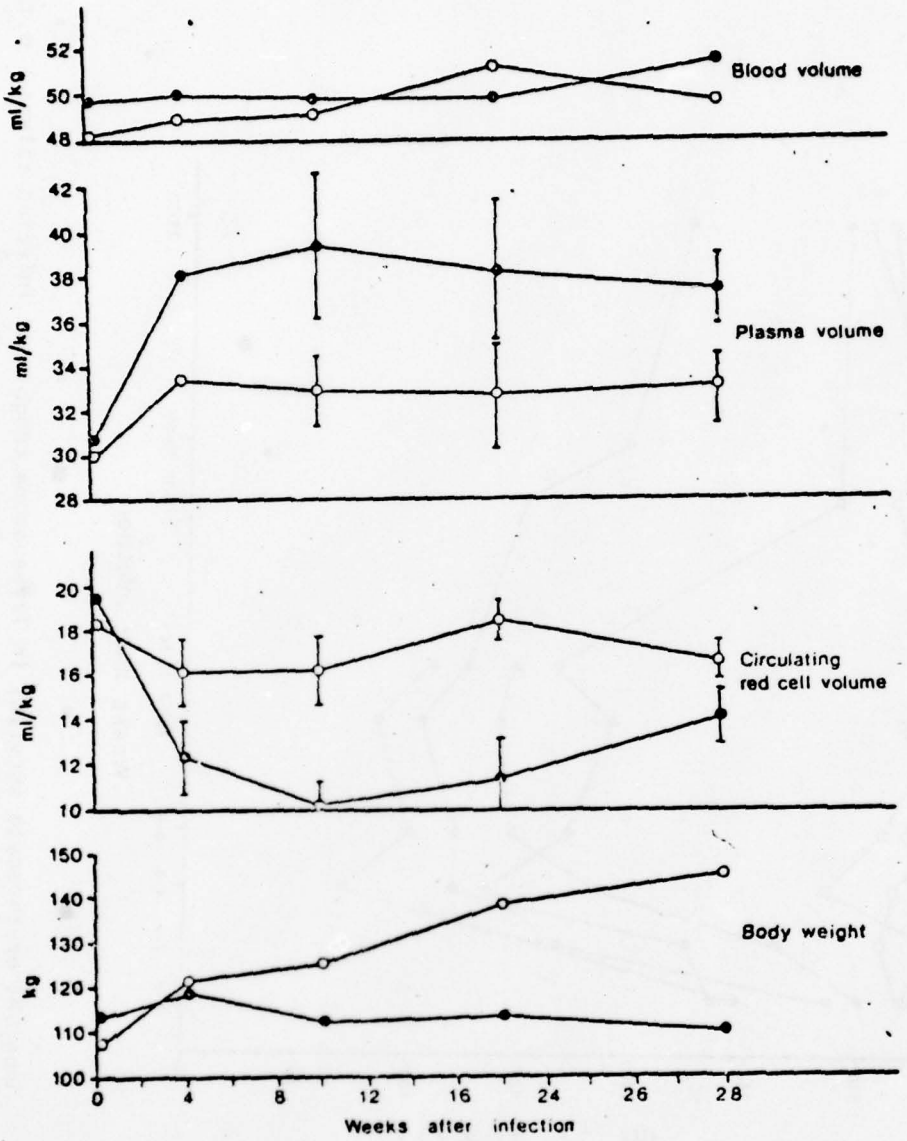


FIG. 3. Volume and weight changes during Trypanosoma congolense infections in calves.

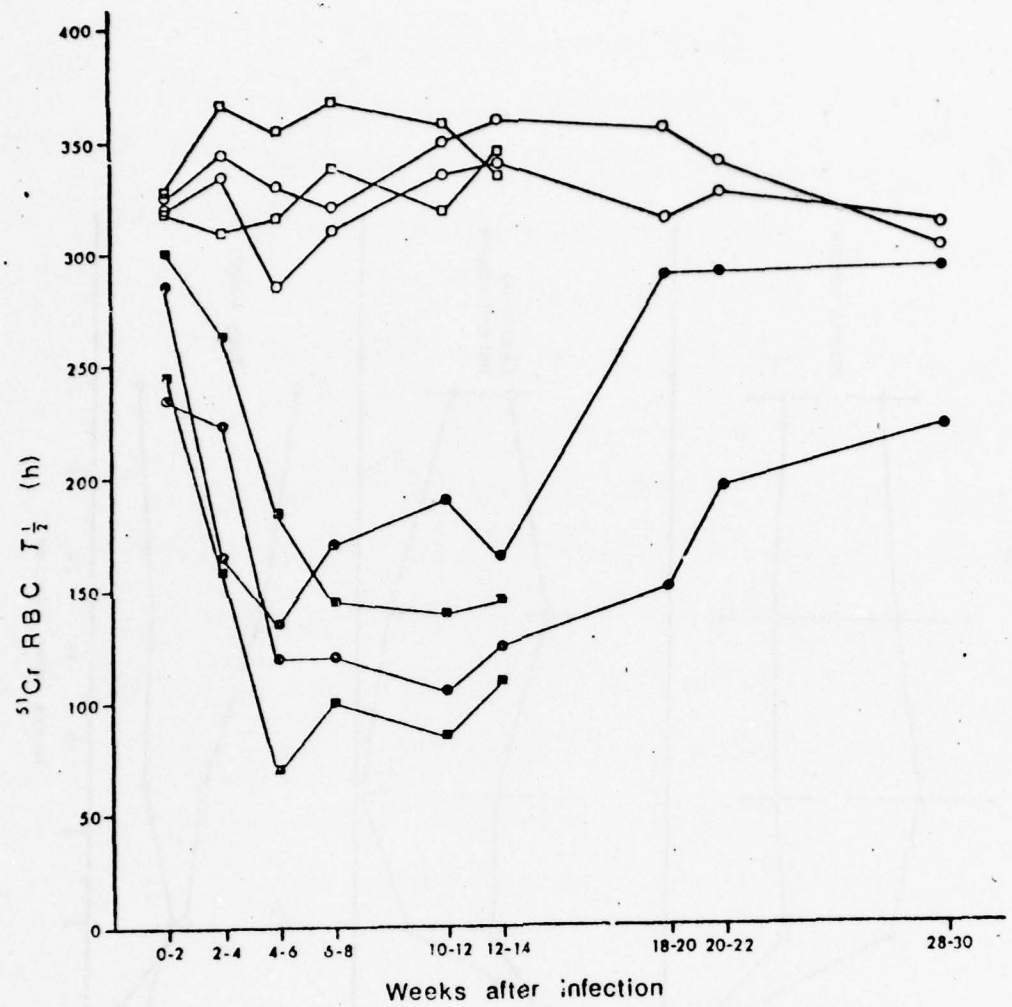


FIG. 4. Apparent erythrocyte survival in Trypanosoma congolense infected calves. o, □, controls; ●, ■, infected (first and second experiments, respectively).

TABLE 1

THE AVERAGE* DAILY FECAL AND URINARY EXCRETION OF ISOTOPE (EXPRESSED IN ML. OF LABELLED ERYTHROCYTES) BY T. CONGOLENSE INFECTED (4) AND CONTROL CALVES (4).

GROUP	FECAL EXCRETION			URINARY EXCRETION		
	WEEKS AFTER INFECTION			WEEKS AFTER INFECTION		
	0-4	4-8	10-14	2-4	6-8	12-14
INFECTED	$2.4^{+}_{-}0.3$	$2.6^{+}_{-}0.6$	$2.2^{+}_{-}0.3$	$77.7^{+}_{-}6.6$	$227.2^{+}_{-}131.0$	$215.6^{+}_{-}117.6$
CONTROL	$2.0^{+}_{-}0.6$	$2.4^{+}_{-}0.4$	$2.6^{+}_{-}2.7$	$39.5^{+}_{-}2.7$	43.0^{+}_{-} 11.8	47.4^{+}_{-} 4.3

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6436	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	139
b. CONTRIBUTING				
XXXXXXXXXX	CARDS 114F			

11. TITLE (Procode with Security Classification Code)

(U) Microbial Genetics and Taxonomy

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	4	341
	CURRENT 79	4	359

19. RESPONSIBLE DOD ORGANIZATION

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ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Rapmund, COL Garrison

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Wohlhieter, J.A. DA

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procode EACH with Security Classification Code)

(U) Vaccine; (U) Enteric Bacteria; (U) Antigens (U) Virulence; (U) Salmonella; (U) Plasmids

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procode text of each with Security Classification Code.)

25. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.

24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.

25. (U) 77 10 - 78 09 Mouse protection conferred by both intraperitoneal and oral immunization with a live Escherichia coli hybrid expressing Salmonella typhi somatic antigens against death from challenge with a virulent S.typhimurium hybrid expressing S.typhi antigens was significant, but inferior to that conferred by immunization with live S.typhi. Six lactose catabolizing plasmids from Salmonella strains clinically isolated in widely separated geographical regions of the U.S.A. were found to be identical in molecular size, and to share all or most of their nucleotide sequences. Hybrids were constructed between coliphage lambda and Salmonella phage P22 in which up to 40 per cent of the P22 genome can be inserted replacing portions of the lambda genome. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

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Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 139 Microbial Genetics and Taxonomy

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CPT D. J. Kopecko, Ph.D.

Description

1. Mouse protection conferred by both intraperitoneal and oral immunization with a live Escherichia coli hybrid expressing Salmonella typhi somatic antigens was significant but inferior to that conferred by immunization with live S. typhi.

2. Six lactose catabolizing plasmids from Salmonella strains clinically isolated in widely separated geographical regions of the United States were found to be identical in molecular size, and to share all or most of their nucleotide sequences.

3. Hybrids were constructed between coliphage lamba and Salmonella phage P22 in which up to 40 percent of the P22 genome was inserted, replacing portions of lambda genome.

Progress

1. Mouse protective capabilities of Escherichia coli hybrids expressing Salmonella typhi antigens.

a. We previously reported using mouse virulent Salmonella typhimurium hybrids expressing S. typhi antigens as challenge organisms in vaccinated Swiss Webster white mice to test the protective capabilities of living and non-living typhoid vaccines, administered either intraperitoneally (i.p.) or orally (Annual Report, WRAIR, 1973, 1974, 1976, 1977). Of the various vaccines examined, live S. typhi administered i.p. is the most effective in protecting mice against death when challenged with the S. typhimurium hybrid. Our primary concern, however, is with evaluating those vaccines against S. typhi that are suitable for human use. We are particularly interested in examining in this assay system the protective capabilities of presently used, as well as potential, human typhoid vaccines. We were, therefore, especially intrigued by the report of Kiefer et al. (1) that mice immunized i.p. with an Escherichia coli hybrid expressing the somatic antigens of S. typhi were significantly protected against death when challenged with our S. typhimurium hybrid which expresses S. typhi antigens.

As an E. coli hybrid of this type would seem most promising for use as a safe, live, oral human typhoid vaccine, we were interested in comparing the protection it would confer in our assay system, administered orally as well as i.p., with that conferred by live S. typhi. Also we wished to see whether the addition of the S. typhi Vi antigen to this E. coli hybrid (by conjugal gene transfer) would enhance that protection.

b. The E. coli parent strain, F464, (serotype O8: K^-: H^-) and E. coli hybrid F1061 were provided by G. Schmidt. E. coli hybrid F1061 is the product of a mating between the S. typhi Hfr strain WR4000 and E. coli F464; as the consequence of replacement of the rfb gene cluster of the E. coli recipient with rfb cluster of the S. typhi donor, E. coli hybrid F1061 expresses the somatic (O) antigens 9,12 of S. typhi, and lacks E. coli O8 specificity. E. coli hybrid WR3078 was generated by a mating between S. typhi Hfr WR4000 and a melibiose non-utilizing (mel) mutant of E. coli hybrid F1061; it was selected for receipt of the S. typhi mel genes, to which the structual genetic determinants of the Vi antigen are closely linked, and it expresses the S. typhi Vi antigen in addition to the S. typhi somatic antigens 9,12. The derivation of the S. typhimurium hybrid H42, used as the challenge organism, has been described previously (Annual Report WRAIR, 1973,1974); it expresses the 9,12, Vi, and d antigens of S. typhi, and has a mean lethal dose of less than 50 organisms. The classical Ty2 strain of S. typhi is, of course, well-known.

c. Organisms to be used as live vaccines in the protection experiments were harvested in phosphate buffered saline, washed three times, appropriately diluted, and mixed 1:1 with a sodium bicarbonate solution (Travenol, 5% injection U.S.P.) just prior to inoculation. Swiss Webster white mice (40 per group), HPB strain, random bred, 16 to 18 g were administered the live organisims either i.p. or orally. Mice immunized i.p. received a dose of 10^7 organisims suspended in 0.5 ml of solution; the groups immunized orally (doses suspended in 0.5 ml of solution and introduced into the stomach with a bent, 25 guage, 2-inch needle with the point removed and end smoothed off) received either a dose of 10^7 organisms, or a dose of 10^9 organisms. All animals were challenged i.p., five weeks after immunization, with 2500 organisms (0.5 ml) of S. typhimurium hybrid H42. Survivors were counted after 21 days.

d. As shown in Table 1, i.p. administration of live S. typhi Ty2 resulted in an 87.5% survival of the mice challenged with S typhimurium hybrid H42, compared with a 62.5% survival among the group immunized i.p. with live E. coli hybrid F1061. The E. coli hybrid WR3078, expressing the Vi antigen in addition to its S. typhi 9,12 somatic antigens, showed no improvement over E. coli hybrid F1061, with only 55% of the immunized mice surviving the challenge.

The failure of Vi antigen addition to enhance the protective capability of a hybrid expressing the S. typhi somatic antigens is similar to our earlier observations with acetone-killed S. typhimurium hybrids as vaccinating strains, and it is consistent with all of our previous findings with regard to the Vi antigen in that we have yet to discover any role for it in the protection conferred in this system. The E. coli parent strain F464, although ranking lowest, as expected, in this protection comparison, also conferred, suprisingly, significant protection, with 42.5% of the i.p. inoculated mice surviving challenge (Table 1). Nevertheless, in several repeated experiments with the parental and hybrid strains, the 20% difference in survival percentage seen here between E. coli F464 and the hybrid F1061 was maintained consistently, confirming the importance of the S. typhi somatic antigens in the protection observed in this system.

e. We had observed previously a 42% survival among mice orally immunized with 10^7 live S. typhi and challenged with S. typhimurium hybrid H42. As seen in Table 2, this figure is almost exactly what we obtained with 10^7 live Ty2 administered orally in the present study. However, at that dose level with live orally administered F1061, although protection was still significantly better ($P>0.01$) than the control group, only 32.5% of the inoculated mice survived challenge. Previously, we have not used higher oral dose levels of live S. typhi than 10^7 because a number of mice die at higher dosages, particularly at 10^9. However, as the E. coli hybrids can be used safely at the 10^9 dose, we went to the higher dosage with live Ty2 as well, in order to make the comparison. As shown in Table 2, oral administration of 10^9 live S. typhi Ty2 raised the level of protection (% survival) to 67.5%, and with the 10^9 oral dose of live E. coli hybrid F1061, the survival figure rose to 47.5%. The E. coli hybrid expressing the Vi antigen, WR3078, again showed no improvement over F1061, with only 40% of the orally immunized (10^9 dose) mice surviving. Once again, at the 10^9 dose, the E. coli parent strain, F464, exhibited a significant ($P<0.01$) level of protection, with 32.5% of the mice in that group surviving.

f. It is evident that the protection conferred by both i.p. and orally administered live E. coli F1061, although significant in these experiments, is nevertheless, inferior to that conferred by live S. typhi Ty2. It is also evident that addition of the Vi antigen to this hybrid (creating E. coli hybrid WR3078) did not enhance its protective capability. Furthermore, in considering the potential usefulness of hybrid F1061 as a live, oral, human typhoid vaccine, it should be noted that a substantial percentage of the overall ability of that hybrid to confer protection in the present experimental system derived from the parent strain, F464, itself, and it seems unlikely that this result would be reflected similarly in the human situation. Overall, the results of these experiments suggest that an attenuated S. typhi strain would be a more effective live human immunizing agent than an E. coli hybrid.

The problem, of course, is the undersirable potential of the S. typhi mutant for producing a systemic infection in the vaccinated human, should back mutation occur.

2. Molecular analyses of plasmids responsible for lactose catabolism in Salmonellae.

a. The atypical lactose fermentation character of six separate clinical isolates of different Salmonella strains, obtained from the stools of diseased humans, has been shown by us previously to reside on self-transmissible extrachromosomal genetic elements (Annual Report WRAIR, 1969,1974). Our past studies focused on the genetic and a brief molecular characterization of these extrachromosomal elements (plasmids). The lactose fermentation trait in each of the six strains was shown to reside on a conjugative plasmid which could transfer itself at a frequency of about 10^{-3} per donor cell per hour or mobilize chromosomal gene transfer from 1 in every 10^7 to 10^8 donor cells per hour. For molecular characterization, radioactively-labelled plasmid deoxyribonucleic acid (DNA) from each strain was purified in cesium chloride-ethidium bromide gradients and then analyzed for molecular size and nucleotide composition. Initial molecular size estimates indicated that the plasmids were of a size between 30 and 50 megadaltons, and all were found to exhibit a 50% guanine + cytosine base ratio.

b. The genetic and physical similarities of these six plasmids indicated that some nucleotide sequences must be shared by them. More recently developed techniques allowed us to examine these plasmids in more molecular detail. Sufficient quantities of purified plasmid deoxyribonucleic acid (DNA) could not be obtained from the original Salmonella hosts or from some Escherichia coli transconjugant hosts, although these strains act as normal conjugal plasmid donors. Since these lactose degrading plasmids have been shown to promote chromosome mobilization, it appears that these plasmids rapidly integrate into the host chromosome in recombinationally proficient strains. To prevent integration and obtain physcially antonomous plasmids for DNA extraction, these plasmids were conjugally transferred to E. coli that are chromosomally deficient both in lactose utilization and general recombination (i.e. lac, recA) or that contained a genetic deletion which included the host lac genes (i.e. Δlac). Plasmid DNA was obtained in sufficient quantities from these latter E. coli presumably because plasmid integration was prevented from occurring.

c. Plasmid DNA was purified from Triton X-100 treated lysozyme induced spheroplasts by CsCl-EtBr gradient centrifugation and subsequent dialysis. Purified DNA was then analyzed for molecular size and relatedness to the other lac plasmids. Molecular sizes were calculated from plasmid contour length measurements obtained with the electron microscope.

All 6 plasmids were found to be identical in molecular size (~41 megadaltons), despite having been isolated from several different Salmonella species that had been obtained from widely separated geographical regions of the United States. Furthermore, these lac plasmids were isolated over a 10 year period between 1955 and 1965 (See Table 3). Identical genetic behavior as well as nucleotide composition and molecular size of these plasmids indicates a close physical interrelatedness. To determine intermolecular homology, these plasmids were cleaved with both the EcoRI and HindIII restriction endonucleases and the specific fragments generated were separated by agarose gel electrophoresis. All plasmids contained apparently identical endonuclease generated fragments, indirectly demonstrating that these lac plasmids share all or most of their nucleotide sequence.

d. These data stongly suggest that in the U.S. there is a widely disseminated plasmid that controls lactose catabolism in Salmonella. The occurence of this plasmid in many different Salmonella strains warrants further examination of other more recently isolated strains for similar lac plasmids (work that is in progress) and a closer study of other lac plasmid controlled properties to see if pathogencity is plasmid associated. Since most adult humans are lactase deficient but lactose tolerant, perhaps these plasmids confer upon the bacterial host the capability to outgrow other non-lactose utilizing organisms in an environment highly enriched in lactose.

3. λimmP22dis: a hybrid of coliphage λ with both immunity regions of Salmonella phage P22.

a. We previously reported the isolation of an unusual Salmonella typhimurium hybrid sensitive to both coliphage λ and Salmonella phage P22. This hybrid bacterium, constructed by mating an Escherichia coli K-12 Hfr donor with an S. typhimurium recipient, is an excellent host for achieving genetic recombination between λ and P22. In our previous reports, we described two broad classes of hybrid phages, those which are structurally λ-like, but have immC region of P22, and those which are like P22, but have acquired the immunity region of λ. Although λ and P22 have gross similarities in functional organization, these phages have a striking difference in their genetic control of immunity. Unlike λ which has a single immunity region known as the c region, two regions are necessary for establishment of immunity to P22. These two regions controlling P22 immunity are referred to as the immC and the immI loci and are widely separated from each other on the P22 genome. In this report, we describe several λimmP22 hybrids which differ in the amount of P22 genome acquired, including one which has obtained both P22 immunity regions.

b. The experimental procedure for obtaining λimmP22 hybrids involves the superinfection with P22 of a λ lysogen of E. coli-S. typhimurium hybrid WR4028. With this procedure, hybrids are then selected for inheritance of the immC marker of P22, by plating on the rough P22-resistant, λ lysogenic derivative of hybrid WR4028. This strain, WR4027 (λ)adsorbs and plates only λ which have acquired the immunity of P22. The λimmP22 hybrids thus obtained were purified on WR4027 and scored for the inheritance of various P22 markers which have replaced the functionally related λ genes. The varying extent of genetic recombination between the parental phages was indicated by the isolation of three different λimmP22 hybrid phages. All three of the hybrids showed a replacement of at least c through P genes of λ with functionally related P22 genes. Hybrid 1 acquired a P22 segment extending from the erf gene through the gene 12. Hybrid 2 had a larger P22 segment which contains the P22 int gene in addition to the markers present in hybrid 1. The third hybrid, which we have designated λimmP22dis carried the immI and the immC regions of P22, both of these loci being involved in the P22 system of bipartite immunity. In order to isolate this hybrid type, the lysate, which results after superinfection of P22 on WR4028 (λ), must be plated on the doubly lysogenic strain WR4027 (λ,λimmP22). When this was done, small clear plaques similar to those of λimmP22 were isolated at a frequency of 10^{-12}. On the basis of their tail antigen characterization and ability to plate on a P22 resistant bacterial strain lysogenic for both λ and λimmP22, the λimmP22dis hybrid exhibits the immunity system of P22. This is indicated by the fact that the immunity patterns of P22 and the λimmP22dis hybrid are identical in all the instances where the two could be compared. These results indicate that λimmP22dis carries the P22 immI region as well as the P22 immC region.

c. The approximate length of P22 DNA that was substituted in λimmP22 hybrids was estimated by backcross experiments between these hybrids and various P22 derivatives. The frequency of recombination between various markers was determined after mixed phage infection of WR4028 by plating on WR4028 resistant to λ to select recombinants. The presence of individual markers in the hybrids was verified by marker rescue experiments. In addition, we employed the more direct method of electron microscope heteroduplex analysis to determine the actual physical length of the recombined segment in the individual hybrids. This procedure makes possible the correlation of the results of the genetic analysis with the quantitative heteroduplex findings. An electron microscope heteroduplex between λimmP22 hybrid 1 and wild-type λ reveals which sections of the hybrid phage genome show homology with the DNA of λ. Portions of the λimmP22 DNA molecule lacking homology λ DNA represent the P22 gene segment substituted in the λ genome.

d. In the heteroduplex formed between hybrid 1 and λ a relatively small P22 region, amounting to about 16% of the genome, inserted close to the right end of λ is observed as a single stranded region of non-homology. Heteroduplexes prepared with hybrid 2 revealed that a somewhat larger block of P22 genes, totaling about 22% of the λ genome, was substituted in this hybrid. Considerably more extensive substitution of P22 genes was observed in heteroduplexes of λ*imm*P22*dis* and λ, wherein approximately 40% of the central region of λ was replaced by P22 genes.

e. The non-homologous region in λ*imm*P22 hybrid 1 extends from a map position of 66 to 82 on the percentage scale of the map of the λ genome. The two regions of non-homology are connected by a small segment of homologous DNA. In λ*imm*P22*dis* hybrid 2, the P22 segment extends from map position 60 to 82 on the λ chromosome. Again, two regions of non-homology, connected by a short segment of homologous DNA are evident. The λ*imm*P22*dis* phage DNA has a non-homologous region that starts at map position 42 and extends to position 82. Three regions of non-homology in this hybrid are separated by two small regions of homology. The non-homologous chromosomal region of this hybrid, extending further leftward on the λ genome than those of the other two hybrid types, goes well beyond the site of the λ *att* gene and thus contains both *imm*I and *imm*C regions of P22. The results of the heteroduplex studies are in good agreement with the results of our genetic experiments.

f. Based on our genetic mapping and heteroduplex studies, we would expect that the λ*imm*P22*dis* hybrid should posses the *S. typhimurium* somatic O-1 antigen conversion gene *al* of P22 as well as tail gene *9* of P22. To determine the presence of the *al* gene, the three hybrid types were used to lysogenize WR4028, and such lysogens were tested for somatic antigen O-1 conversion of the host. As expected, only the WR4028 (λ*imm*P22*dis*) lysogen expressed *Salmonella* somatic antigen O-1 indicating that this hbyrid contains the P22 *al* gene. To determine whether tail gene *9*, which is located near the *al* gene, is expressed during λ*imm*P22*dis* replication, we employed an *in vitro* self-assembly method. P22 heads without tails were obtained by growing a temperature sensitive P22 tail gene mutant *ts9*, at elevated temperature. When the head preparation was added to a lysate of λ*imm*P22*dis* and then plated on *S. typhimurium*, a one million-fold increase in P22 plaque-forming activity was observed. These observations establish that tail gene *9* of P22 is being expressed by the λ*imm*P22*dis* hybrid, although the protein coat and tail of this hybrid phage are those of λ.

Summary

1. An Escherichia coli hybrid, F1061, expressing S. typhi somatic antigens 9,12, and a derivative of this hybrid, E. coli hybrid WR3078, expressing the S. typhi Vi antigen in addition to somatic antigens 9,12, were compared with S. typhi Ty2 in experiments to test their ability, as live vaccines, to protect Swiss white mice against death from challenge with a mouse virulent S. typhimurium hybrid expressing the S. typhi antigens 9,12, Vi and d. When the live, vaccinating organisms were administered intraperitoneally, 87.5% of the mice immunized with S. typhi Ty2 survived challenge, as compared with 62.5% of those immunized with E. coli hybrid F1061, and 55% of those inoculated with E. coli hybrid WR3078. When these live organisms were administered orally, at a dose of 10^9, 67.5% of the mice immunized with S. typhi Ty2 survived challenge, as compared with 47.5% of those immunized with E. coli hybrid F1061, and 40% of those administered E. coli hybrid WR3078. Thus, the protection conferred by E. coli hybrid F1061 expressing only the S. typhi somatic antigens, although significant in this system, was inferior to that conferred by S. typhi Ty2, and the addition of the S. typhi Vi antigen to this hybrid did not enhance that protection.

2. Six lactose catabolizing plasmids, initially observed to be harbored in six separate clinical isolates of different Salmonella strains, were conjually transferred to E. coli strains that were genetically deficient both in lactose utilization (lac) and general recombination (recA), or that contained a delection which included the lac genes. Plasmid deoxyribonucleic acid (DNA) was obtained from these E. coli strains by Triton X-100 treatment of lysozyme-induced spheroplasts followed by purification on cesium chloride-ethidium bromide gradients. Molecular sizes were calculated from plasmid contour length measurements obtained with the electron microscope. The nucleotide sequence homology among the plasmids was assessed by examination of their fragmentation patterns following digestion with restriction endonuclease EcoRl and slab gel electrophoresis in agarose. All six plasmids were found to be identical in molecular size (~ 40 megadaltons) despite having been isolated from several different Salmonella species that had been obtained from widely separated geographical regions of the United States. Furthermore, these plasmids contained 6 identical EcoRl-generated and 4 identical Hind III-generated fragments, indicating that the six plasmids share all or most of their nucleotide sequences. These results suggest that there is in the United States a widely disseminated ancestral plasmid that controls lactose catabolism in Salmonella.

3. Genetically marked λ and P22 phages were recombined in Escherichia coli-Salmonella typhimurium hybrid WR4028, a host sensitive to infection by both of these phages. Hybrid phages that acquired the immC region of P22, but retained the genes for the λ protein coat were selected on WR4027 (λ), a λ-immune, P22-resistant derivative of WR4028. In these λimmP22 hybrids at least c through P genes of λ were replaced with functionally related P22 genes. Phage recombinants with more extensive regions of the P22 genome were selected on the double lysogen WR4027 (λ,λimmP22). One such hybrid, λimmP22dis, was determined by heteroduplex analysis to contain approximately 40% of the P22 genome. Genetic studies established that λimmP22dis possesses the two widely separated immunity control regions of P22 (immC and immI), and that these loci are expressed in E. coli K-12 lysogenic for λimmP22dis. In addition, λimmP22dis contains the P22 al locus responsible for somatic O-1 antigen conversion in Salmonella. Although the λimmP22dis phage particle has the λ head and tail, the phage genome also carries P22 tail gene 9 as evidenced by the production of free P22 tails. It also has the P22 att site as indicated by the integration of the λimmP22dis prophage near the proA locus on the bacterial chromosome.

Table 1

Protective capabilities of intraperitoneally administered live vaccines in mice challenged with S. typhimurium hybrid H42[a]

Vaccinated with	Survivors/ No Inoculated [b]	% Survival
S. typhi Ty2	35/40	87.5
E. coli hybrid F1061	25/40	62.5
E. coli hybrid WR3078	22/40	55.0
E. coli parent F464	17/40	42.0
Control	1/40	2.5

a
The vaccinating dose was 10^7 organisms, and the animals were challenged i.p. after 5 weeks with 2500 organisms (0.5 ml) of S. typhimurium hybrid H42.

b
Protection conferred by all of these strains is significantly better ($P<0.005$) than the control.

Table 2

Protective capabilities of orally administered live vaccines in mice challenged with S. typhimurium hybrid H42[a]

Vaccinated with	Dose	Survivors/ No. Incoulated	% Survival
S. typhi Ty2	10^9	27/40[b]	67.5
	10^7	17/40[b]	42.5
E. coli hybrid F1061	10^9	19/40[b]	47.5
	10^7	13/40[c]	32.5
E. coli Hybrid WR3078	10^9	16/40[b]	40.0
	10^7	12/40[c]	30.0
E. coli parent F464	10^9	13/40[c]	32.5
	10^7	8/40	20.0
Control		2/40	5.0

a Animals were challenged i.p. after 5 weeks with 2500 organisms (0.5ml) of S. typhimurium hybrid H42.

b Protection is significantly better ($P<0.005$) than the control.

c Protection is significantly better ($P<0.01$) than the control.

Table 3

Summary of Lactose-Fermenting Plasmids Studied

Plasmid Designation	Originial Host Strain	Locale of Isolation	Year Isolated
pWR20	Salmonella tennessee	1507-65 California	1965
pWR22	" "	545-57 New Jersey	1957
pWR26	" "	999-57 New York	1957
pWR28	" "	886-57 New Jersey	1957
pWR32	" senftenberg	1688-55 Illinois	1955
pWR50	" typhimurium	4349-64 Massachusetts	1964

All bacterial strains were isolated from human feces and were kindly supplied by W.H. Ewing of the Center for Disease Control in Atlanta, Ga.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 139 Microbial Genetics and Taxonomy

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6450	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	140
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Procede with Security Classification Code)

(U) Military Hematology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008800 Life Support 002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	10	397
FISCAL YEAR CURRENT 79	10	417

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
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NAME: C. C. Daughaday, MAJ, MC DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Coagulation; (U) Malaria; (U) Blood; (U) Anemia

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define the hematologic pathophysiology of infectious diseases of military importance, and of trauma, burns and shock, and to identify modalities to restore nemostasis.

24. (U) Procedures include biochemical, immunologic, and cell culture methods; in vitro cell-free and membrane-dependent systems; large and small laboratory animal models; and studies of human subjects.

25. (U) 77 10 - 78 09. Pyridoxal kinase was shown to be absent in the malaria parasite. Several purine salvage patnway enzymes were shown to increase markedly in monkey erythrocytes infected with P. knowlesi and in cultured human erythrocytes infected with P. falciparum. Purine and pyrimidine nucleoside and base profiles were obtained in malaria-infected erythrocytes. Pyridoxal was demonstrated to be a potent antisickling agent which may be clinically useful. In vivo fibrin and fibrinogen degradation were examined in detail in 20 normal people before and after exercise. A radioimmunoassay for early peptides released in degradation of the alpha chain of fibrinogen is in the final stages of development. The role of factor VIII complex in thrombosis and in the post-trauma and post-operative states was examined. Certain lectins, such as con A were shown to stimulate erythroid cell growth in vitro whereas others, such as WGA were inhibitory; evidence was obtained to suggest the possibility of a lymphocyte mediator. A humoral inhibitor of stem cell growth was demonstrated in a patient with erythroid aplasia. Detailed studies were performed to examine the precise chemical nature of the covalent bond between C3 and cell membranes. Superoxide dismutase and catalase were snown to be protective against pulmonary oxygen toxicity at intermediate oxygen tensions, but to have little effect at high oxygen tensions. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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PROJECT 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 140 Military Hematology

Investigators

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Description

Basic and clinical studies were performed to investigate the functions of blood and blood-forming organs. These focused on four specific areas: (1) study of normal blood cell metabolism and its alteration by disease or by chemical agents, (2) examination of biochemical and immunologic determinants of hematopoiesis and of intracellular parasite growth, (3) examination of blood cell surface membrane composition and function in normal and disease states and (4) study of the structure, function, and chemical modification of hematologically important proteins.

Progress

1. Study of normal blood cell metabolism and its alteration by chemical agents

Metabolic studies during the past year have focused on five areas: (a) studies of the role of pyridoxine metabolism in malaria infection; (b) studies of purine metabolism in malaria-infected erythrocytes; (c) metabolic studies of bone marrow erythroid precursors; (d) examination of the effects of oxygen tension, superoxide dismutase, and catalase on lipid peroxidation of lung cell suspensions; and (e) study of metabolic changes in platelets with exercise.

a. Studies of the role of pyridoxine metabolism malaria infection

Study of the possible protective role against falciparum malaria of low erythrocyte pyridoxal kinase (PLK) levels was continued during this year. Previous work performed by our department in Nigeria, in collaboration with investigators from the University of Ibadan and from the Laboratory of Parasitic Diseases, NIAID, suggested that the relatively high frequency of low red cell PLK activity among Blacks may have been selected for by falciparum malaria. In these studies, performed during the summer of 1976 and published in 1978, the red-cell glucose-6-phosphate dehydrogenase (G-6-P.D.) activity and red cell pyridoxal-kinase (P.L.K.) activity of 27 Nigerian children with severe Plasmodium falciparum parasitemia were compared with those of 26 healthy Nigerian children and 6 White adults. The mean P.L.K. activity of the malaria patients was similar to that of the Whites but significantly higher than that of the Nigerian controls. Correction for reduced mean red-cell age in patients was made by comparing the P.L.K.: G-6-P.D. ratio for those with stable G-6-P.D. phenotypes. The mean P.L.K.: G.-6-P.D. ratio was the same for malaria patients and adult Whites but significantly higher than that for the Nigerian controls. During the summer of 1977, frozen hemolysates from 150 Nigerian children were collected (100 from children with severe malaria infection, 50 from controls). Work is currently in progress to isolate and characterize the low and high pyridoxal kinase variants in red cells, so that these samples can be genotypred.

The hypothesis that plasmodia depend on host erythrocytes for pyridoxal kinase was examined in detail. Blood was drawn from animals with a 20% to 80% parasitemia, white cells and platelets were removed, erythrocytes were lysed with 0.2% saponin, the parasites were separated from the hemolysate by centrifugation, and PLK was assayed in both fractions. Isolated intact and lysed P. gallinaceum (from 6 chickens), P. falciparum (from 2 aotus monkeys), and P. knowlesi (from 1 patas and 2 rhesus monkeys) exhibited no PLK even when assayed with cell counts from 10 to 20 times those used for erythrocytes. Erythrocyte fractions from chickens, aotus monkeys, and the patas monkey showed reproducible

PLK. Plasmodia did not inhibit PLK, since the PLK of infected cells lysed by hypotonic media was the same as the erythrocyte fraction from saponin lysis. Plasmodia did not bind or destroy the product of PLK, since at least 97% of added pyridoxal phosphate was recovered after incubation with isolated parasites. In two experiments glucose-6-P-dehydrogenase activity (G6PD) and pyruvate kinase activity (PK) were assayed in the cell fractions. All G6PD was found in the supernatant and more PK was found in the parasite fraction, suggesting that separation of erythrocyte cytoplasm from parasites was efficient and did not destroy enzymes. We concluded that these species of plasmodia do not contain significant levels of PLK, and that dependence of plasmodia on host erythrocyte PLK might explain the protective effect of low erythrocyte PLK trait for humans exposed to falciparum malaria infections.

b. Studies of purine metabolism in malaria-infected erythrocytes

Studies of purine metabolism in malaria-infected erythrocytes focused on two areas: utilization of high performance liquid chromatography to examine purine and pyrimidine nucleotide, nuceloside, and base profiles in uninfected, malaria-infected, and drug-treated infected erythrocytes; and examination of levels of purine salvage and interconversion enzymes in uninfected and malaria-infected monkey and human erythrocytes.

Until this past year, our studies of levels of substrates involved in purine and pyrimidine metabolism have focused on the mono-, di-, and trinucleotides. During the past year, our focus has broadened to include the purine and pyrimidine nucleosides and bases as well. A sensitive and reproducible method for detection and quantitation of purine and pyrimidine bases and nucleosides was developed using reverse-phase HPLC. The present method allows assay of 14 compounds at the nanogram level. Work is in progress which indicates that the deoxy- and methyl forms of these purine and pyrimidine compounds can also be simultaneously assayed by the HPLC procedure. By putting a fluorescent detector in series with the UV-detector, an ehanced resolution of the chromatographic separation is achieved. Compounds with fluorescent properties, (i.e., uric acid, tryptophan etc.), masked by UV

background are resolved from a single HPLC run. Current efforts are directed toward: (1) determination of basal levels of purine and pyrimidine nucleosides and bases in plasma, whole blood, and blood components (e.g., erythrocytes, platelets), with emphasis on human, monkey, and rat species; and (2) examination of changes in purine and pyrimidine nucleosides and bases during malaria infection. Reproducible profiles have been established for perchloric acid (PCA) extracts of plasma and whole blood from three species (human, monkey, rat). Preliminary studies of nucleoside and base profile alterations during malaria infection indicate the presence of a new peak eluting close to adenosine in chromatograms of infected animals. Efforts are currently in process to identify this peak as the deoxy or methyl form of adenosine.

Activities of purine salvage and interconversion enzymes have been determined in erythrocytes from rhesus monkeys both before and during infection with P. knowlesi. We are attempting to correlate these enzyme activities with respect to levels of purine nucleotides, nucleosides, and bases obtained on other aliquots of the same specimens.

c. Metabolic studies of bone marrow erythroid precursors

The de novo pathway of purine biosynthesis is known to be deficient in the human mature erythrocyte. Whether the complete de novo synthetic pathway is present at any time in human erythropoiesis is not known, but circumstantial evidence suggests that the maturation of the red blood cell might be accompanied by the loss of a number of steps in the de novo pathway. Specifically, the rabbit reticulocyte possesses full capacity for the de novo synthesis of nucleotides. During maturation to an erythrocyte, this capacity is lost through deletion of the ablity to synthesize IMP from simple precursors. The distal portion of the pathway, involving the ability to synthesize AMP from IMP through adenylosuccinic acid (AdSA) is retained. In the human, a different situation obtains. The portion of the pathway distal to IMP is not intact in the human because of deletion of the enzyme adenylosuccinate synthetase (AdSS), responsible for the conversion of AdSA to IMP. The enzyme adenylosuccinate

lyase (AdSL), responsible for the conversion of Ad SA to IMP, continues to be present.

In view of the alterations in biosynthetic capability attending red cell maturation in the rabbit and because of the importance of the de novo synthetic pathway to disease models and to an understanding of the importance of hepatic versus extrahepatic purine biosynthesis, we investigated this pathway in human erythroid precursors in two ways. Using human bone marrow obtained from normal ribs removed at operations (for exposure, as with certain types of nephrectomy), activity of adenylosuccenate synthetase and incorporation of labeled formate and glycine into purines were determined in whole bone marrow, and preliminary attempts were made to demonstrate these two synthetic activities in isolated erythroid precursors. Adenylosuccinate synthetase was demonstrated in whole human bone marrow for the first time, but technical difficulties have so far prevented us from obtaining good data showing the presence or absence of this enzyme activity in isolated erythroid precursors. Similarly, a small but significant incorporation of labeled glycine and formate into purines was shown in whole marrow but not isolated erythroblasts. Recent improvements in our technique make it likely that we will be able to examine these synthetic activities in isolated erythroid cells definitively in the near future.

d. Examination of the effects of oxygen tension, superoxide dismutase, and catalase on lipid peroxidation of lung cell suspensions

Lung cell suspensions are sensitive to lipid peroxidation, one of the phenomena thought to be responsible for the toxicity of oxygen to the lung. We have found that lipid peroxidation in vitro is enhanced by oxygen in a dose dependent fashion. In addition we have found that the antioxidant enzymes, superoxide dismutase and catalase, inhibit lipid peroxidation at intermediate tensions of oxygen (300-400 torr) but begin to lose effectiveness at high tension (600 torr).

e. Study of metabolic changes in platelets with exercise

Platelet content of ADP, ATP, and serotonin, as

well as aggregation in response to a variety of agents, was examined in ten healthy, fasting adult males exercised on a treadmill using the Balke protocol. Blood samples were drawn before exercise (C), immediately after exercise (E) and one hour after exercise (R). Mean heart rates were 68 at rest and 194 at the termination of exercise. Mean time on the treadmill was 22.3 minutes.

Results (means) of platelet studies were:

	C	E	R
nMADP/10^8 plt	4.4±0.7	2.1±1.1	4.6±2.2
nM ATP/10^8 plt	5.8±1.6	6.8±1.4	6.6±1.5
µg serotonin/10^8	11.8±1.7	13.6±1.9	12.7±4.4

There were no significant changes in platelet aggregation in response to ADP, epinephrine, collagen or ristocetin.

The magnitude of the decrease in platelet ADP content (52%, $p<0.001$) after exercise cannot be accounted for by exercise-induced thrombocytosis, even if all platelets released during exercise were devoid of ADP.

The rapid recovery of platelet ADP levels to control values would suggest that the decrease in platelet ADP with exercise is due to a transient metabolic alteration rather than to a loss of platelet ADP storage granules.

2. Biochemical and immunologic determinants of hematopoiesis and of intracellular parasite growth

This research area evolved as a result of our department's interest in hematopoiesis, and our collaborative efforts with members of the Immunology and Virology Departments in the Division of Communicable Diseases and Immunology. The specific areas on which we have focused during the past year include: (a) biochemical determinants of intraerythrocytic malarial parasite growth in culture; (b) effects of vitamin B6 deprivation on erythroid cell proliferation and heme synthesis enzymes; (c) modulation of erythroid cell proliferation by immunologic mediators and cellsurface active agents; (d) dengue fever virus-monocyte inter-

actions; and (e) relationship between interferon production and virulence of Semliki forest virus.

a. Biochemical determinants of intraerythrocytic malarial parasite growth in culture

In collaboration with Maj David Haynes (Department of Immunology, Division of Communicable Diseases and Immunology), we are examining the role of a number of metabolic pathways, particularly vitamin B6 metabolism and purine metabolism, in P. falciparum growth in human erythrocytes, using Maj Haynes's culture system.

Under the aegis of WHO research agreement T16/181/MZ/8, Mr. Vincent C.N. Okoye from the University of Ibadan, Nigeria, is working in our department as a visiting scientist. He is examining the effect of reduced levels of the enzymes pyridoxal kinase (PLK) and glucose-6-phosphate dehydrogenase (G-6-P.D.), and of the substrate ATP on the growth of P. falciparum in human erythrocytes in vitro. Each of these polymorphisms has been shown to be low in a substantial percentage of American Blacks. One hundred thirty five healthy subject have been screened for biochemical polymorphisms.

The groups of subjects consist of fifty Black American men, twenty-two Black American women, thirty-seven Caucasian American men, twenty one Caucasian American women, and six people from other backgrounds (two Nigerian men; one man from India; two women and one man from the Caribbean). Studies performed on blood from each of these subjects included: (a) quantitative determination of G-6-P.D. activity, and electrophoresis of G-6-P.D.; (b) hemoglobin electrophoresis; (c) measurement of pyridoxal kinase activity; (d) ATP levels, and (e) complete blood group phenotyping. Because it was felt by all investigators involved in this project that first priority should be given to comparison of growth of P. falciparum in erythrocytes with high pyridoxal kinase levels with growth in erythrocytes with low pyridoxal kinase levels, this study is being done first. Six Black American men with low erythrocyte pyridoxal kinase levels (less than 0.50 mIU) but no other observed polymorphisms (i.e., normal G-6-P.D. levels; HbA; normal ATP levels) were selected for comparison with six Black American men with high

erythrocyte and no other observed polymorphisms. Blood was drawn from each of these twelve subjects, and the initial biochemical data confirmed by repeating the determinations. Growth of P. falciparum in erythrocytes from subjects in each of the two groups is currently under examination. A B6 deficient medium (special order GIBCO 1640, made without B6) is used in these studies; the only B6 available is a small amount bound to serum albumin and an even smaller amount in the erythrocytes. This medium has been shown to be capable of supporting intraerythrocytic parasite growth in previous studies performed by Maj Haynes using erythrocytes from randomly selected Caucasian donors. Following completion of this culture study, a comparable study will be performed comparing growth in G-6-P.D. deficient hemizygotes, G-6-P.D. deficient heterozygotes, subjects heterozygous for G-6-P.D. deficiency, and subjects with normal G-6-P.D. levels.

Following the report by Kortynyk et al (Biochemistry 15:5458, 1976) that analogues of pyridoxal and of pyridoxal phosphate in which the 4-CHO group is replaced with CH=CH2 inhibited growth of mouse Sarcoma 180 and mammary adenocarcinoma TA3 in cell culture, and that the growth inhibitory effects of these analogues correlated well with their effects on pyridoxal kinase and pyridoxine phosphate oxidase in cell-free systems, we arranged through the Division of Experimental Therapeutics to have four of these analogues made. These four compounds are: (a) 4-vinyl-4-deformyl-pyridoxal (WR238952 AA); (b) 4-(2-trans-methylvinyl)-4-deformylpyridoxal hydrochloride (WR 239287 AA); (c) 4-(2-trans-methylvinyl) -4-deformylpyridoxal 5' -phosphate (WR 240275 AA); and (d) 4-(2-cis-methylvinyl)-4-deformyl-pyridoxal 5' phosphate (WR 240242 AA). The inhibitory effects of each of these compounds is currently being examined using the P. falciparum in vitro culture system with B6 deficient medium.

Activities of purine salvage and interconversion enzymes have been determined in human erythrocytes infected with P. falciparum in culture. A parasite-specific isozyme of one of these enzymes has been identified, and is being purified for further characterization, with our ultimate objective the finding of an agent active against the parasite enzyme but not the host enzyme.

b. Effect of B6 deprivation on erythroid cell proliferation and heme synthesis enzymes

The effect of dietary pyriodoxine deprivation on erythroid cell proliferation and heme synthetic enzyme activity was examined in nine dogs maintained on a B6-free diet. Erythroid colony forming units (CFU-E) were determined in bone marrow specimens obtained from seven of the B6-dificient animals at times when the plasma and erythrocyte PLP levels indicated profound deficiency in this vitamin and when the animals were anemic. Dose-response curves to pyridoxal ($3X10^{-4}M$, $3X10^{-5}M$, $3X10^{-6}M$) were obtained in each case. The data show that while there are less *in vitro* CFU-E present in the marrow from B6 deficient dogs, the dose response to pyridoxal *in vitro* is generally similar to that in normal dogs; i.e., the optimum growth is seen at $3X10^{-4}M$ and $3X10^{-6}M$. Two B6 deficient dogs had almost no CFU-E on baseline studies and did not respond to pyridoxal at any doses used. These two animals were severely anemic when studied, but they did recover completely when B6 was added to their diet, as did the other five B6-deficient dogs. It is postulated that these two animals may have had a block in an earlier stem cell compartment (e.g., CFU-S or BFU-E) as a result of severe prolonged B6 deficiency. Delta-aminolevulinic acid synthetase (ALA-S) and heme synthetase (HS; ferrochelatase) activities were measured in bone marrow aspirates from all nine B6-deficient dogs at times when they were B6-replete and when they were B6-deficient and anemic. Enzyme activities were determined both in the presence and in the absence of added pyridoxal-5'-phosphate. Erythroblast ALA-S activity was diminished to approximately 50% of control levels; activity could be restored to normal by *in vitro* addition of pyridoxine to the marrow lysate. On the other hand, heme synthetase activity remained normal in all but the more severely anemic B6-deficient animals; in the bone marrow of these animals, in vitro addition of PLP did not increase the heme synthetase activity significantly. Thus, the sequence of changes occurring with B6 deprivation appears to be: (a) diminished plasma and erythrocyte PLP levels, associated with normal CFU-E, normal Hct, diminished ALA-S activity (one-half normal value; corrected to normal by *in vitro* PLP addition); (b) early anemia, associated with more significantly lowered ALA-S activity (not fully corrected with *in vitro* PLP addition), variable decrease in heme synthetase activity, and diminished CFU-E (correctable with

pyridoxal added in vitro): and (c) late anemia, associated with markedly diminished ALA-S and HS activitites and markedly decreased CFU-E, none of which are responsive to added B6.

c. Modulation of erythroid cell proliferation by immunologic mediators and by cell-surface active agents

Because eosinophilic fasciitis (EF), a rare immunologic disease characterized by muscle weakness and eosinophilic infiltrates, has been known to terminate in bone marrow aplasia, an immunologic mechanism for this aplasia was sought. A 67 year old man at WRAMC with biopsy-proven eosinophilic fasciitis, anemia, and thrombocytopenia was studied. During hospitalization, the patient's platelet count fell to 10,000 and his Hct to 31. His bone marrow biopsy was 30% cellular. Platelet antibody studies by quantitative complement lysis-inhibition showed 61.3 fg IgG/platelet. Plasmapheresis was performed to decrease antibody titer, and post plasmapheresis the titer was 14.0 fg/platelet. Plasma clot cultures to determine bone marrow CFU-E and BFU-E were done. The patient was noted to have less BFU-E and CFU-E than normal control. Addition of post plasmapheresis serum did not alter growth in either patient or control marrow when compared to serum from normal control. However, with pre-plasmapheresis serum CFU-E/10^5 cells fell in the normal from 260.3 to 156.0 ($p<.001$) and in the the$_5$patient form 149.8 to 119.3 ($p<.02$). Also, BFU-E/10^5 cells fell in the normal from 63.1 to 31.0 ($p<.001$); in the patient it fell from 23.5 to 18.5 ($p<.1$). Co-culture of normal marrow showed no suppression of CFU-E. Growth of BFU-E in similar cultures was 40-75% of expected values. Although anemia was mild, thromobocytopenia resulted in several bleeding episodes. Of all therapies used, only Velban-loaded platelets produced a sustained increase in platelet count. We concluded from the data that this patient with EF demonstrated platelet antibodies and a serum inhibitor of his own and normal erythroid stem cells. Although co-culture studies suggest cell-cell suppression of BFU-E the latter may be the result of previous sensitization to HLA antigens. Humoral suppression of hematopoietic elements may lead to decreased hematopoiesis in this disease.

Studies on the interaction between lections and

CFU-E in plasma clot culture have been continued, and all studies for an initial publication on this topic have been completed. Three lections with known receptors on mature red cells Concanavalin A (Con A), Phytohemagglutinin (PHA), and Wheat Germ Agglutinin (WGA) were studied for their in vitro effects upon bone marrow erythroid colony forming units (CFU-E) from C3H mice in plasma clot cultures. Dose response curves indicated that all three lectins had significant effects upon CFU-E in doses as low as 1-2.5μgm/10^6 cells. WGA suppressed growth at all doses. PHA suppressed CFU-E at low and high doses with normal growth at 5-10μgm/10^6 cells. Con A stimulated CFU-E at low doses and suppressed growth at or above 25μgm/10^6 cells. Since PHA and WGA have been shown to interact with erythropoietin (Epo), preincubation and binding studies with 125 I labeled lectin were done to see if this mechanism completely accounted for suppression of CFU-E at low lectin doses. Greater suppression was seen following preincubation of cells with lectin than when amounts equivalent to that bound after preincubation were added directly to the culture medium, even though 94-95% of the lectin bound after preincubation eluted off the cells during culture. Trypan blue studies showed no evidence of cytotoxicity after 30 min incubation with any of the lectins at the doses used. CFU-E following preincubation with Con A were equal to controls. Further studies involved addition of monosaccharides to cultures without lectin to test whether the lectins were masking a cell surface saccharide needed for Epo-cell interaction. Of the saccharides tested, sialic acid alone inhibited CFU-E. We concluded that the lectins tested affect CFU-E by direct effects upon the marrow cells as well as interference with Epo-cell interaction (WGA and PHA). These cells directly affected may involve the CFU-E itself or an intermediate cell.

d. Denque fever virus-monocyte interaction

Studies of denque fever virus-monocyte interactions have been carried out in collaboration with Dr. Walter Brandt, of the Department of Virus Diseases, Division of Communicable Diseases and Immunology. Sensitivity of the receptor on monocytes for Denque fever virus to trypsin has been demonstrated. Following treatment with trypsin, monocyte cultures become nonpermissive for dengue virus replication. The permissive state is

reconstituted by Dengue immune serum, but only partially restored by nonimmune serum. Studies designed to demonstrate the importance of the Fc fragment of IgG antibody and its interaction with monocyte Fc receptor are underway.

e. Relationship between interferon production and virulence of Semliki Forest Virus

Differences in interferon induction and sensitivity between two strains of Semliki Forest Virus (SFV)-A774 (avirulent in several mouse lines, and L10 (highly lethal) were examined. Duplicate wells of L929B fibroblasts were infected with L10 and A774 viruses at several different MOI's. At 24 hours, no interferon was detected in the supernatant from cells induced by the avirulent A774, whereas the induction of interferon by L10 correlated with the MOI. At 48 hours. interferon was produced in response to A774 but still in lower amounts than that produced by L10. Studies of induction of interferon by L10 and A774 at various temperatures (30°, 35°, 39°C) suggested that the lack of interferon induction at 37°C by A774 was not a result of a temperature sensitive step which would be reversed at 30°C. Comparison of interferon production with viral growth indicated that L10 virus produced more interferon than A774, yet the latter virus grew to a higher titer. Additional studies suggested that L10 might also be more sensitive to interferon than is A774. On the basis of experimental results available so far, the differences in virulence between L10 and A774 cannot be explained by interferon production alone.

3. Examination of blood cell surface membrane composition and function in normal and disease states

Our department's studies of the role of blood cell surface membrane composition and function during the past year have included (a) studies of pulmonary alveolar macrophage function, (b) studies of biochemical composition and function of lymphocyte membranes, and (c) studies of platelet membranes.

a. Studies of pulmonary alveolar macrophage function

During the past year, studies have been initiated

on the effects of N-formyl-methionyl phenylalanine (FMP) on the function of alveolar macrophages. FMP is one of several synthetic compounds that have been shown to be chemotactic for both neutrophils and macrophages, and is probably responsible for the chemotactic activity of supernatants of bacterial cultures (bacterial factor). Many eukaryotic cells, including most gram negative bacteria, synthesize their proteins with a formylated methionine residue on the amino terminus. The studies of the effects of FMP on alveolar macrophage function have provided important insights into the interaction of these cells with bacteria.

Our findings to date indicate that adherence to glass of macrophages exposed to 100 μM FMP is increased approximately two-fold after 72 hours of incubation at 37 degrees (in RPMI 1640 with 20% heat inactivated fetal calf serum). There is also a marked stimulation of movement as shown by measurements of random migration and the appearance of elongated spindle cells on the glass coverslips (control 10.75%; 100 μM FMP 92.5 after 4 hours). Other studies underway include FMP stimulated chemoluminescence and alveolar macrophage production of interferon.

b. Biochemical composition and function of lymphocyte membranes

Binding studies with 5 purified plant lectins were used to investigate membrane alterations in lymphocytes from patients with chronic lymphatic leukemia (CLL). Compared to normal human B lymphocytes, CLL lymphocytes had fewer receptors for E-phytohemagglutinin, wheat germ agglutinin, and concanavalin A, and more receptors for L-phytohemagglutinin. Receptors for Ricinus communis agglutinin were the same on both normal and CLL cells. Since the lectins bind to complex carbohydrates on the cell surface, these data suggested that the carbohydrate composition of CLL membranes differed from that of normal lymphocytes. To investigate this point, exposed membrane sialoglycoproteins on intact cells were radiolabeled by a combination of mild periodate oxidation followed by reduction with NaB^3H_4. Analysis by SDS polyacrylamide gel electrophoresis of extracts from the radiolabeled cells indicated that the CLL cell membranes contained the same protein components as normal lymphocyte membranes, but that these components were generally

less heavily glycosylated in CLL. To investigate mechanisms responsible for the altered glycosylation of CLL cell membrane components, we examined glycosyltransferase activities in cells from CLL patients and normal donors. Compared to normal cells, CLL cells demonstrated decreased ability to transfer N-acetyl glucosamine from UDP-N-acetyl glucosamine to appropriate exogenous or endogenous glycoprotein acceptors. We conclude that CLL lymphocytes are characterized by a specific pattern of altered cell surface glycoproteins which can be detected by lectin binding studies. These alterations may be related to decreased glycosyltransferase activity in the CLL lymphocyte.

Glycosyltransferase activities in human lymphocytes stimulated with the plant mitogens E-phytohemogglutinin (E-PHA) or concanavalin A (con A) were determined, and the results were compared with activities found in resting lymphocytes. Compared to resting lymphocytes, con A-stimulated lymphocytes possessed an enhanced capacity to transfer the sugars, sialic acid, galactose, and N-acetylglucosamine from their respective nucleotide donors to both endogenous cellular acceptors and added exogenous glycoprotein acceptors. The enhanced glycosyltransferase activity induced by con A was not inhibited by puromycin despite effective inhibition of de novo protein synthesis, indicating that synthesis of new glycosyltransferase enzymes is not necessary for the observed increases in glycosylation activity. By contrast to the findings in con A-stimulated cells, the corresponding glycosyltransferase activities of E-PHA-stimulated lymphocytes did not differ from those of unstimulated lymphocytes. These data indicate that individual plant lectins have different effects on the biosynthesis of complex saccharides by cultured human lymphocytes.

c. Studies of platelet membrane

A group of twelve cancer patients with clinical signs of hypercoagulation were studied with a battery of in vitro clotting parameters to include: platelet aggregation, plasma partial thromboplastin time, fibrinogen content, factor VIII complex activity, platelet surface sialic acid, and platelet surface sialyltransferase activity. These patients demonstrated accelerated coagulation, increased factor VIII antigen and Ristocetin cofactor, and enhanced ADP platelet aggregation. Platelet

exogenous sialyltransferase activity was increased in cancer patients (117.6 + 14 p mole/10^9 platelet as compared to controls 59.0 + 4.3 pmole/10^9 platelets, $p<0.01$). Platelet exogenous sialyltransferase activity and platelet aggregation were inhibited by aspirin.

4. Study of the structure, function and chemical modification of hematologically important proteins

Our studies on the biochemistry and physiology of hematologically important proteins have focuses on four major areas: (a) studies of fibrinolysis; (b) studies of complement; (c) use of radioimmunoassays as tools for study of the biosynthesis, chemical modification, and kinetics of a number of glycoprotein hormones and other hormonal substances; and (d) examination of the anti-sickling effect of pyridoxal and other pyridoxine vitamers.

a. Studies of fibrinolysis

Studies of the hemostatic changes associated with exercise have been completed in 20 subjects. In addition to acceleration of clotting assays and elevation of factors VIII and V, increased fibrinolytic activity, including elevated fibrinogen-fibrin associated antigen (split products of fibrin or fibrinogen, FDP-fdp), was demonstrated.No significant fibrinogen Aα-chain degradation or changes in the ability of fibrinogen to clot and form crosslinks were seen.

A radioimmunoassay for the earliest degradation products from fibrinogen/fibrin (i.e., those from the Aαchain) has been developed. The assay is sensitive to nM quantities of digestion products and is being applied to samples from exercise studies.

Fibrin has been shown to crosslink much more rapidly when red cells are present in the coagulum. The effect appears to be due to a sulfhydryl dependent property of the external surface of the red cell.

Analysis of fibrinogen/fibrin degradation by isoelectric focusing and SDS polyacrylamide slab gel electrophoresis (two dimensional electrophoresis) is in progress. This method should be a very sensitive way to determine Aα-chain degradation of fibrinogen/fibrin.

Urinary protein is now being analyzed for fibrin degradation products using several methods: two dimensional electrophoresis, radioimmunoassay, and cyanoethylation. Cyanoethylation tests specifically for the presence of the E (-glutamyl) lysine covalent crosslinks of crosslinked fibrin.

b. Studies of complement

In collaboration with Dr Brian Tack (American National Red Cross) and Dr Alan Schechter (Laboratory of Chemical Biology, NIAMDD, NIH), radioimmunoassay studies examining the conformation of C3 and its activation fragments C3b, C3c, and C3d, have continued and are now near completion. Two specific observations were made in this study: (1) Antigenic responses to antibodies raised in sheep to human C3 are elicited in C3 and fragments C3b and C3c, but not in C3d. (2) The biphasic % bound vs fragment concentration curves indicate that the C3b and C3c fragements have surface conformations different from the same regions in native C3.

c. Use of radioimmunoassays as tools for study of the biosynthesis, chemical modification and kinetics of a number of glycoprotein hormones and other hormonal substances

Because of the rapidly developing interest of the hematology community in using radioimmunoassay as a probe for studying both the conformation (see above) and the enzymatic interconversion of such proteins as coagulation factors and complement, we have supported, as part of our department's effort, certain studies on hormone biochemistry and physiology whose approach we feel is directly applicable to problems in complement and/or coagulation biochemistry.

Radioimmunoassays were developed for 3',5'-diiodothyronine (3',5'T2) and 3'-monoidothyronine (3'T1). The antisera to these hormones were quite sensitive, with the lower limits of detectability being 2.0 ng/dl and 2.5 ng/dl, respectively. The mean intra- and interassay coefficients of variation were <3% and <24%. Of numerous thyroid analgues tested for cross reactivity, only rT3 slightly cross reacted in the 3',5'T2 assay and only 3,3'T2 cross reacted in the 3'T1 assay. Employing these assays, eleven normal subjects and 7 pregnant

women had serum $3'T_1$ levels below the limits of detectability of the assay (0.5 ng/dl), whereas 17 hyperthyroid patients had elevated levels of $3'T_1$, with the mean (± SD) values being 6.5 ± 3.0 ng/dl. Serum $3'T_1$ levels were present in all cord sera measured (7.3 ± 2.3 ng/dl; n=19), and the highest levels of $3'T_1$ observed were in 38-40 week gestation amniotic fluid specimens (15.4 ± 8.4 ng/dl; n=20). Compared to other iodothyronines, it appears that a relatively low proportion of $3'T_1$ is bound to circulating proteins, as the mean percent dialyzable $3'T_1$ in 12 normal subject was 5.7 ± 1.2%. An oral dose of $3'T_1$ (120 µg) given to 2 euthyroid individuals resulted in peak serum levels of 28 ng/dl two hours after ingestion. After i.v. administration $3'5'T_2$ to two athyreotic patients (one hypothyroid, the other euthyroid on replacement T_4), $3'T_1$ levels rose from undetectable levels to 20 ng/dl. Conclusions from this study were that $3'T_1$ is routinely detectable in the serum of hyperthyroid but not normal individuals, with even higher levels present in cord sera and amniotic fluid, and that <u>in vivo</u> conversion of $3',5'T_2$ to $3'T_1$ may occur.

Employing a radioimmunoassay for 3'5'T2, it was determined that 83 healthy individuals had a mean (±SE) serum 3',5'T2 concentration of 5.0 ± 0.3 ng/dl; thyrotoxic subjects (n=12) had a mean 3',5'T2 level that was elevated to 10.8 ± 0.8 ng/dl whereas each of 6 hypothyroid subjects had undetectable (< 2 ng/dl) concentrations. Athyreotic patients (n=8) receiving 0.4 mg T4 daily had serum 3',5'T2 concentrations of 15.0 ± 3.0 ng/dl and fasting was associated with an increase in serum 3',5'T2 to 6.9 ± 0.6 ng/dl from a basal level of 4.4 ± 0.4 ng/dl in the fed state ($p<.01$). Despite the fact that reverse T3 levels may be elevated in amniotic fluid and that reverse T3 is expected to represent the major source from which extrathyroidal 3'5'T2 arises, 3',5'T2 levels were low in amniotic fluid being undetectable (<2 ng/dl) in 9 of 19 samples with the mean (±SE) 3',5T2 concentration in the 10 detectable samples being 5.4 ± 1 ng/dl. These data indicate 3',5'T2 is probably derived from peripheral conversion. Furthermore, these observations suggest that situations associated with elevated reverse T3 levels (e.g., thyrotoxicosis, fasting) also have increased 3',5'T2.

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To investigate outer ring deiodination of T4 and

reverse T3 (rT3), an RIA was employed to measure 3'T1 generation from 3'5'T2 in subcellular fractions of rat liver. In vitro 5'-deiodination of 3',5;T2 to 3'T1 was found to be rapid, heat labile, pH dependent and inhibited by PTU (10^{-4}M). The high lability to outer ring deiodination of rT3 and 3',5'T2 (pKA of 4'-OH = 6.7) at the optional pH in our system in contrast to the metabolic stability of 3,3'T2 and 3'T1 (pKA of 4'-OH=8.4) may indicate a requirement for an intact 4'-OH and/or specificity for degree of inner ring iodine substitution.

d. Examination of the antisickling effect of pyridoxal and other pyridoxine vitamers

To test the antisickling activity of pyridoxal, we compared the oxygen affinity and the percent sickling at low PO_2 of untreated erythrocytes with values for cells from the same blood sample incubated with pyridoxal, glyceraldehyde, or pyridoxine. Pyridoxal increased oxygen affinity much more than glyceraldehyde. 20 mM pyridoxal and glyceraldehyde had equivalent antisickling activity. At PO_2 levels above 20 mm Hg, both agents reduced sickling to less than 20%. In samples examined by electron microscopy, pyridoxal reduced the percent sickled cells and the percent cells that contain hemoglobin S fibers by the same amount (from 74 to 3%) Pyridoxine had no effect on oxygen affinity or sickling. Pyridoxal reacts with intracellular hemoglobin to increase oxygen affinity, which inhibits hemoglobin S polymerization and sickling.

PROJECT 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 140 Military Hematology

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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11. TITLE (Precede with Security Classification Code)

(U) Pathogensis of Renal Disease of Military Importance

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23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To investigate mechanisms for maintaining fluid, electrolyte and hemodynamic homeostasis in response to disease, injury and environmental stresses of military significance, such as acute renal failure, shock, infectious disease, heat stress, and gastrointestinal disorders, in order to provide rational basis for prevention and treatment.

24. (U) Clearance methods, dialysis, isotope dilutions, experimental models, in vivo micropuncture, in vitro renal microperfusion, membrane transport, tissue culture, radioimmunoassay, light and electron microscopy, and chromatography.

25. (U) 7710-7809 The tubuloglomerular feedback mechanism, important in the pathogensis of acute renal failure, was further defined. Juxta glomerular renin release, which presumably controls feedback, was found to be sensitive to chloride delivery to the tubule rather than to urinary sodium or flow rate. Examination of the interrelationships of sodium excretion revealed that prostaglandins play a significant role only during states of stress (anesthesia, hypotension). Renin levels in thoracic duct lymph were seen to parallel levels of peripheral blood during volume expansion and hypotension suggesting a significant lymphatic role in renin metabolism. Mercuric chloride, (used extensively in the production of acute renal failure), was shown to produce reversible inihibition of vasopressin mediated water movement in the toad bladder and time-dependent permeability to inulin suggesting an alternative mechanism for the isosthemuria of acute renal failure and supporting a role for tubular back-diffusion in acute renal failure. Dithiothreitol, (an agent protective against the nephrotoxicity of mercuric chloride), was shown to reversibily inhibit vasopressin mediated water flux in the toad bladder while simultaniously stimulating sodium transport. Renal epithelial cells in tissue culture were found to be utilizeable as a simplified system for studying renal transport and responsiviness to hormones and nephrotoxins. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 77 - 30 Sept 78.

* Available to contractors upon originator's approval

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Project 3M161102BS01 BASIC RESEARCH ON INJURY AND DISEASES

Work Unit 141 Pathogensis of Renal Disease of Military Importance

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Description

Studies are directed at investigations of mechanisms for maintenance of body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance such as acute or chronic renal failure, shock, heat stress, physical stress, infectious diseases and gastrointestinal disorders. The role of adaptive homeostatic mechanisms, including renal and systemic mechanisms, whereby body fluid and solute balance are maintained in the face of stress, has been emphasized in order to provide a rational basis for the development of improved methods for prevention and treatment of altered fluid, electrolyte, and hemodynamic states, and acute and chronic renal failure induced by these alterations.

Progress

1. Acute Renal Failure

a. The mechanism(s) responsible for the pathogenesis of acute renal failure, a disease characterized by the sudden loss of renal function, continues to be the subject of controversy. A majority of recent studies indicate that an alteration in renal hemodynamics, mediated by the renin-angiotensin system, contributes a major role in the near cessation of glomerular filtration and the progressive azotemia observed in acute renal failure (1, 2). A schema for the pathogenesis of the hemodynamic changes in experimentally induced acute renal failure in animals has been developed from previous studies in this laboratory based on the effects of heavy metals such as uranyl nitrate (3) and mercuric chloride (4). The initial event in the sequence involves an alteration in tubular transport function in the early segments of the nephron. This epithelial membrane transport alteration can be characterized by decreased fluid and electrolyte transport as a direct effect of

the nephrotoxin (and perhaps ischemia) on membrane function (5). As a result of this transport defect the fluid delivered distally to the macula densa segment contains a higher than normal chloride load. There is an increase in juxta-glomerular renin activity (6). The increased renin-angiotensin system activity mediates a change in renal hemodynamics by a diminution in total renal blood flow with preferential outer cortical ischemia (7). These hemodynamic alterations result in a decline in glomerular filtration (3). Despite this decline in filtration, the tubular transport defect in the early nephron results in continued delivery of fluid of altered chloride content to the macula densa and the altered hemodynamic changes persist (7). In addition, as cell death occurs in those segments of the nephron exposed to the highest concentrations of nephrotoxins, increase in permeability occurs so that back diffusion of bulk solutes across the tubular basement membrane may result in significant absorption of residual filtrate (8).

b. Although the abnormalities in tubular transport function have been observed in experimental acute renal failure induced by various models, it was not known if they represent the primary alterations as suggested above, or were the secondary consequences of ischemia. Studies were therefore carried out to characterize the effects of mercuric chloride and uranyl nitrate on membrane function. These studies utilized the urinary bladder of the fresh water turtle (Pseudemys scripta) which allowed observations on an intact membrane of mesonepheric origin in an in vitro preparation deprived of alterations in renal or systemic hemodynamics (9, 10). This membrane has been well characterized for its similarity to the mammalian nephron in its ability to transport sodium and secrete hydrogen and in its ability to respond to aldosterone (11). These studies demonstrated that both uranyl nitrate and mercuric chloride inhibited sodium transport across the short-circuted urinary bladder of the turtle when added to the mucosal but not the serosal side. This inhibition could be reversed by the mucosal addition of dithiothreitol, a compourd capable of complexing heavy metals and maintaining monothiols in solution. These studies provide evidence that heavy metals may directly result in alterations in membrane transport similar to those proposed in the schema above and similar to those observed in previous studies (3).

Parallel studies were performed in the urinary bladder of the fresh water toad (Bufo marinus) to assess the effects of heavy metals on membrane permeability (8). This anuran membrane is similar to that of Pseudemys scripta in many respects, with the exception that it behaves much more like the mammalian nephron in its response to vasopressin. These studies, also performed in vitro, allowed characterization of the effect of mercuric chloride

on vasopressin mediated osmotic water flow and on passive permeability to substances of higher molecular weight. These studies revealed that mercuric chloride after short-term incubation (20 minutes) inhibits vasopressin stimulated osmotic water flow in a reversible fashion. Long-term exposure to mercuric chloride (120 minutes) resulted in increase in basal permeability of the membrane to water, irreversible inhibition of vasopressin response, and permeability to solutes which normally do not cross this membrane (inulin, mol. wt. 5,000). These studies suggest that long-term exposure to mercuric chloride, and other heavy metals, may result not only in altered epithelial transport but also in increased basal permeability. These observations therefore support not only the altered transport hypothesis noted above, but also the role of tubular back-diffusion, postulated as important in maintaining the later stages of acute renal failure.

c. Previous studies from this laboratory have demonstrated that dithiothreitol is capable of favorably altering the course of heavy metal induced renal failure and preventing the accompanying natriuresis and enhanced juxta-glomerular apparatus renin activity (12). These effects have been interpreted as resulting directly from inhibition of the effects of the nephrotoxin on tubular transport mechanisms. Little attention has been directed to an alternative hypothesis that dithiothreitol might be binding to specific hormone receptor sites and/or interfering with the effect of the renin-angiotensin system to elicit a renal ischemic response. In this regard several investigators have also suggested an effect of antidiuretic hormone in contributing to the cortical ischemia of acute renal failure (13). Studies recently completed (14) in the urinary bladder of the toad (Bufo marinus) indicate that dithiothreitol produces a competitive, reversible inhibition of antidiuretic hormone-mediated osmotic water movement across this membrane. These observations are compatible with inhibition of the hormone receptor site. Observations in the isolated rabbit aorta indicate that dithiothreitol also interfers with the angiotensin receptor site and inhibits the constrictor response to angiotensin in vitro (15). These observations suggest that dithiothreitol, in addition to inhibiting the effect of heavy metals on membrane transport, as noted in the schema above, may also interfere with the effector limb of the response, namely the intrarenal vasoconstrictor response as mediated by the renin-angiotensin system. Studies are currently in preparation to assess the in vivo effect of dithiothreitol on the vasoconstrictor response to angiotensin infusion and alternatively to determine if dithiothreitol might directly inhibit the release of renin from the juxta-glomerular apparatus in vitro. A model to study this latter effect has recently been developed in our laboratory.

d. Studies have been continued to assess the mechanisms responsible for juxta-glomerular renin release. Although previous studies of the effects of volume contraction or volume expansion on renin release have equated the effect with sodium depletion or repletion, respectively, recent studies suggest that the chloride ion rather than the sodium ion is the responsible species. Studies by Kotchen et al (16) would suggest that plasma renin levels correlate inversely with the delivery of chloride to the macula densa regardless of the accompanying cationic species. Recent observations from this laboratory (6) suggest that juxta-glomerular renin content is also inversely related to distal chloride delivery rather than to sodium delivery. Studies are currently in progress to study these effects directly.

Studies are currently in progress to assess the relative contribution of the renal lymphatics to the overall metabolism of renin. In these studies the thoracic duct is cannulated by the method of Witte et al (17). Following stabilization, lymphatic and venous renin activity were monitored after induction of volume expansion or contraction and both are finally compared to renal juxta-glomerular renin content. Thus far these studies indicate that thoracic duct renin content parallels systemic release and suggests that the lymphatics may contribute significantly to renin metabolism.

e. Effects of the renal vasoactive hormones

Although the renin-angiotensin system appears to be of primary importance in the control of tubular-glomerular feedback and in the initiation of acute renal failure, the kidney synthesizes other vasoactive substances which may interact with the renin-angiotensin system and with prostaglandins which appear to contribute significantly to modulation of renal blood flow, and to sodium and water homeostasis during stress. The systemic infusion of prostaglandins of the E or A series has been shown to be natriuretic and hyperemic. However, these studies have been subjected to the criticism that they do not mimic the effects of endogenous prostaglandin production; renal prostaglandins being currently held to reach their site of action directly via the tubules. For this reason we have chosen to circumvent this problem by continuing to study the effects of the prostaglandin inhibitors, meclofenamate and indomethecin. Previous studies from this and other laboratories (18) have produced conflicting results regarding the role of endogenous prostaglandins on renal blood flow and sodium excretion. These conflicts have been partially resolved since it was determined that considerable differences occurred after prostaglandin inhibition in conscious as opposed to anesthetized dogs (19).

In the conscious dog prostaglandin inhibition has no effect on renal blood flow, however, it reduces renal blood flow in the anesthetized dog suggesting that normally prostaglandins have little role in modulating renal blood flow but that in states of stress prostaglandins are vasodilatory. The effect of prostaglandins on sodium excretion remains somewhat controversial. A recent study suggested that prostaglandin inhibition in the conscious dog produced a natriuresis suggesting that prostaglandins are anti-natriuretic (20). Studies reported from this laboratory (19) have failed to confirm this latter observation and have suggested that prostaglandin inhibition in consicous, volume expanded dogs did not significantly alter sodium excretion. This supports the hypothesis that prostaglandins modulate renal blood flow and sodium excretion only during states of stress. Similar conclusions have resulted from recently reported studies during controlled hemorrhagic hypotension in the conscious dog(20).

f. In addition to studying the mechanisms and reversibility of nephrotoxic acute renal failure, studies have been initiated to develop a model of rhabdomyolysis-induced acute renal failure. Renal failure is associated with the induction of rhabdomyolysis primarily due to exertion, heat injuries, drug intoxication, viral illness and potassium depletion (21). Studies from this laboratory (22) and the surgical research unit (23) have suggested that in addition to inadequate physical preconditioning, mild to moderate potassium depletion may predispose the recruit to rhabdomyolysis and renal failure. Attempts to reproduce exertional rhabdomyolysis in the experimentally potassium depleted animal in the past have been unsuccessful, largely because of the lack of the motivational factor in the animal. In attempting to find a suitable model to study the effects of exertion-induced rhabdomolysis we have utilized the model of the potassium depleted rat trained to exercise in a rotating drum. Preliminary observations thus far indicate that potassium depletion alone does not result in significant rhabdomolysis in this model. We have modified this basic model to include the superimposition of a myotoxic drug on pre-existing potassium depletion. In these studies dietary potassium depletion in induced, as corroborated by plasma potassium levels and balance studies, and the animals are subsequently treated with plasmocid, an 8-aminoquinolone antimalarial. Whereas neither potassium depletion nor plasmocid alone produce significant rhabdomolysis, the combination produced significant rhabdomolysis in all animals studied. This was corroborated by laboratory evidence of elevated serum glutamic-oxalic transaminase and creatine phosphokinase levels, as well as myoglobinuria and light miscroscopic evidence of active muscle necrosis. In addition, azotemia has been noted, accompanied by histologic evidence of tubular degeneration and pigmented tubular casts.

These preliminary observations suggest that this model may be of considerable use in studying the mechanisms of rhabdomolysis-induced acute renal failure.

2. Acid Base Homeostasis

a. The maintenance of extracellular acid-base homeostasis is a major function of the kidney. In appropriate renal responses to shock, sepsis and stress may result in life threatening systemic acidosis or alkalosis. In order to better understand the tubular mechanisms responsible for acid base homeostasis so that more effective therapeutic modalities might be devised studies were continued to further define the metabolic requirements for acidification. The model utilized was the urinary bladder of the fresh water turtle (Pseudemys scripta) in which active urinary acidification has been shown to be dependent upon oxidative metabolism and the substrate glucose. Studies confirmed that inhibition of hydrogen transport by either an imposed pH gradient or by acetazolamide resulted in decreased glucose metabolism via the pentose shunt. Conversely, stimulation of hydrogen transport resulted in increased activity of the pentose shunt. Both of these parameters varied independently of glycolylic metabolism. In addition, the pentose shunt pathway enzyme activity was found to be localized to the mucosal fraction of the bladder which is the cell layer responsible for acidification. These studies may be an important metabolic pathway for hydrogen transport by the turtle urinary bladder.

3. Regulation of Body Water Hoemostasis

The kidney is the major organ responsible for maintaining the constancy of volume and composition of the fluid which bathes and delivers nutrients to the cells of the body. In this regard it functions to maintain the volume and osmotic composition of the body fluids within very narrow limits. Regulations of the osmotic composition (osmolality) depends primarily on the availability of water intake from the environment and the appropriate release, or the suppression of release of arginine vasopressin, the mammalian antidiuretic hormone. The release of vasopressin for the pituitaty is normally under osmotic control. The pituitary responding to changes in osmotic concentration of as small as 1-2% with appropriate release, or suppression of release, of vasopressin. In states of volume depletion or shock, hypotensin through stimulation of carotid baro-receptors may cause release of vasopressin which contribute to maintenance of intravascular volume but which is inappropriate for osmotic regulation and results in hyponatremia and hypoosmolality. In other conditions, such as tumors, infection, central nervous system disease or injury, and or administration of drugs and anesthetics, vasopressin may be

inappropriately released and result in serious retention of water and hypoosmolality, which can cause life threatening metabolic disturbances. Vasopressin also has other effects which may be of importance in body homeostasis. Vasopressin administration results in increased peripheral resistance with reduction in renal blood flow and has been implicated in the production of acute renal failure; it causes release of renal prostaglandins which partially countermand its vascular effects, and it appears to be of major importance in regulating memory (25).

Because of the importance of vasopressin in mediating body water homeostasis and its importance in states of shock, infection and acute renal failure studies have been undertaken to further define its' mechanism(s) of action.

a. The primary recognized function of vasopressin is to increase the permeability of the distal nephron to water resulting in increased osmotically mediated reabsorption of water from the collecting duct. At the cellular level, following binding to its receptor, vasopressin results in an increased adenylate cyclase activity with increased production of cyclic adenosine monophosphate (26). Through stimulation of a protein kinase, cyclic adenosine monophosphate results in phosphorylation of a membrane protein and the formation of increased numbers of microtubules, one or both of which are felt to result in increased permeability of the apical (urinary) surface of the tubular cell to water and to urea. The intracellular content of cyclic adenosine monophosphate is also regulated by a phosphodiesterase which converts it into an inactive form and which is inhibitable by a number of drugs such as theophyllin. The mechanism of attachment of vasopressin to its mucosal receptor has been held in the past to be through a sulfhydryldithiol interchange as it has been demonstrated that agents which are capable of reducing or binding sulfhydryl groups can inhibit one or more of the actions of vasopressin (27). Recent evidence however suggests that a sulfhyryldithiol interchange may not be necessary for maintenance of vasopressin activity and suggests that membrane sulfhydryls are important in receptor function for other reasons, i.e., maintaining structural configuration of the receptor or of aqueous channels. Because sulfhydryl groups are necessary for the cellular action of vasopressin to occur, we undertook a study of the effects of sulfhydryl inhibitors on vasopressin responsiveness. These studies were conducted in vitro in the urinary bladder of the Dominican toad, Bufo marinus, an epithelial membrane well characterized for its responsiveness to vasopressin. This membrane has been previously demonstrated to respond to vasopressin by increasing its permeability to urea and to osmotically induced water flow and by increasing active transport of sodium from mucosal (urinary)

to serosal surface. The effects of dithiothreitol, a compound which reduces dithiols and stabilizes sulfhydryls, was studied on vasopressin mediated osmotic water flow. Mucosal dithiothreitol (10^{3}M) was shown to completely inhibit the effects of submaximal vasopressin (10 mU/ml) on osmotic water flow. Inhibition was reversible either by removal of dithiothreitol from the bathing media or by the addition of supramaximal concentrations of vasopressin. Studies undertaken to evaluate the site of inhibition reveal that dithiothreitol also inhibits the response to theophyllin and to dibutyryl-cyclic adenosine monophosphate suggesting that inhibition occurs somewhere beyond the generation of cyclic AMP. Dose response curves indicate that, while both mucosal and serosal dithiothreitol inhibit vasopressin mediated water movement, a much higher mucosal concentration is required, suggesting that this agent does not act directly on the mucosal epithelium and that sulfhydryl groups other than membrane sulfhydryls are of importance in the ultimate action of vasopressin. At lower concentrations (10^{5}M), which do not inhibit the action of vasopressin, dithiothreitol produces complete and apparently irreversible inhibition of theophyllin mediated water movement suggesting that it interferes with the interaction of theophyllin with phosphodiesterase or acts distal to the formation of cyclic adenosine monophosphate. As dithiothreitol has no effect on vasopressin stimulated water movement at these doses it would suggest that the theophyllin responsive pathway is not necessary to the action of vasopressin in this membrane. Preliminary observations suggest that dithiolthreitol does not affect basal or vasopressin stimulated tissue levels of cyclic adenosine monophosphate.

b. Numerous studies suggest that glucocorticoids exert a significant effect on renal water handling and that deficiency of glucocorticoids results in decreased free water clearance. Despite numerous investigations into the possible mechanisms of glucocorticoid facilitation of renal water handling, no uniform concept of its mode of action has involved. Studies were therefore undertaken in adrenalectomized dogs to further delineate the mechanisms of the glucocorticoid effect. Adrenalectomized dogs were maintained with dexamethasone, DOCA and normal (100meq daily) sodium intakes for two weeks following recovery from surgery. After stabilization on this regimen the animals were rendered glucocorticoid insufficient by withdrawal of dexamethasone for 48 hours prior to study. Each animal was studied in the conscious state after both glucocorticoid withdrawal and again while on glucocorticoids to determine the effects of glucocorticoid withdrawal on free water clearance, glomerular filtration rate, renal plasma flow, renal plasma renin activity and plasma vasopressin. In addition, prior to the termination of each study the animals received the prostaglandin inhibitor meclofenamate, 2mg/kg, to

assess the role of prostaglandins in the mechanism of glucocorticoid action. Glucocorticoid insufficiency resulted in a significant decrease in free water clearance when compared to the glucocorticoid repleted state, 10.4 ± 2.6 vs. 22.9 + 1.9 ml/min ($p<.01$). This was unrelated to changes in mean arterial blood pressure, glomerular filtration rate, or renal plasma flow. There were no differences in minimal urinary osmolality in each group and plasma renin and vasopressin were consistantly suppressed in both groups. Meclofenamate produced significant inhibition of urinary prostaglandins in the glucocorticoid repleted groups and did not further suppress the already suppressed levels in the insufficient group. Prostaglandin inhibition had no effect on free water clearance in either group. These studies suggest that decreased free water clearance with glucocorticoid insufficiency is independent of changes in blood pressure, glomerular filtration rate, renal plasma flow, antidiuretic hormone or changes in renal prostaglandin production. They support the contention of other investigators that other factors limiting distal fluid delivery (increased proximal reabsorption) are of importance in the mechanism of impaired water handling in glucocorticoid insufficiency.

4. Phosphate Homeostasis

As a consequence of stress or acute or chronic renal failure, major changes in concentration of plasma phosphate may occur. Changes in plasma phosphate levels have important implications for wound healing, bone metabolism and maintenance of cellular integrity. The regulation of plasma phosphate levels by renal reabsorption and excretion of this complex anion is a major physiological control mechanism. Although previous studies from this laboratory have characterized the various sites along the nephron involved in phosphate transport (28), little is know about the active cellular process involved.

Studies have been designed to examine the influence of both extracellular pH and buffer conditions on trans-epithelial phosphate transport. The studies were carried out in a simplified transporting epithelia, the isolated bladder of the fresh water turtle. Previous studies have established that anuran membranes can be acceptable models for the study of phosphate transport in vitro (29, 30).

Net mucosal to serosal flux of phosphate was determined as the difference between undirectional ^{32}P fluxes in the absence of transepithelial electrochemical gradients. With 5mM phosphate in bicarbonate free ringers at pH 8.4 Jp was 21.8 + 7.4 nanM/hr/8cm^2. There was a slight increase in Jp with isohydric addition of 10mM HCO_3 to the mucosal bath. Addition of 5% Co_2, which reduced pH

to 7.3 did not affect Jp. At pH 8.4, Jp was not affected by addition of ouabain, 2-4 dinitrophenol, acetazolamide, parathyroid hormone or 3'-5' cyclic adenosine monophosphate. Jp increased progressively as phosphate concentration was raised between 0.5 and 10 mM at pH 8.4. At pH 6.2 in the absence of exogenous Co_2 and bicarbonate, Jp was undetectable. With 2.5 mM HCO_3 and 5% Co_2 at pH 6.5 Jp was 61.3 $\pm$ 16.0 nanM/hr/8cm^2 and decreased to 30.6 $\pm$ 1.6 nanM/hr/8cm^2 when pH was raised to 7.2 by increasing bicarbonate concentration to 10 mM. At pH 6.5, Jp was not affected by acetazolmide, parathyroid hormone or 3'-5' cyclic adenosine monophosphate but was inhibited by ouabain and 2-4 dinitrophenol. Jp increased progressively as phosphate concentration was raised between 0.5 and 10 mM at pH 6.5.

The results suggest the existence of two separate pathways for transepithelial phosphate transport. The transport of HPO_4^- at acid pH is CO_2 dependent and inhibited by ouabain and dinitrophenol. This pathway may be further characterized by examination of potential cation coupled transport suggested by inhibition of Jp associated with inhibition of transepithelial sodium transport (31). In contrast, the transport of $HPO_4^=$ at alkaline pH is not CO_2 dependent or inhibited by ouabain or dinitrophenol. This pathway may be further characterized by the use of inhibitors specific for anionic transport pathways such as DIDS or SITS (32).

5. Transport by Epithelial Cells in Culture

Recent work from this laboratory has stressed the role of cellular and membrane function in acid-base homeostasis (24), phosphate homeostasis (33) and acute renal failure (34). Accumulated evidence from a number of laboratories has led to the suggestion that the study of transport by epithelial cells in culture can increase understanding of both normal homeostatic mechanisms and pathophysiologic states (35). Cell culture offers obvious advantages over other in vivo or in vitro approaches: (1) Cloning permits the study of a homogeneous population of a single cell type, (2) large amounts of homogeneous material can be grown for analytic or cell fractionation studies, (3) the environment of cells in culture can be completely regulated, not only during in vitro measurements but for long periods before study and (4) genetic techniques can be used. The initial efforts of this laboratory in conjunction with the LKEM of NHLBI have been to establish primary cell lines and continuous cultures of transporting epithelia and to demonstrate that the cells in culture maintain the differentiated functional properties of interest. Two approaches have been attempted; (1) primary culture of epithelial cells from anuran tissue; (2) use of established lines of mammalian kidney cells in culture.

a. Development and study of primary and continuous cultures of epithelial cells from anuran membranes.

The urinary bladder of the toad, Bufo marinus, has been used extensively to study active transepithelial sodium transport and the regulation of transport and permeability by hormones. Primary cell cultures of epithelial cells from toad urinary bladder have been grown and continuously cultured for over 25 sequential passages and an estimated 50 generations (36). Cells are harvested by incubating the bladders as sacs in Ca++ and Mg++ free ringers containing 2 mM EGTA. The cells are collected and resuspended in Ca++ and Mg++ free ringers without EGTA and added to culture medium in plastic dishes incubated at 28°C in a humidified atmosphere of 4% CO_2 in air. Culture medium is a mixture of seven parts Coon's modification of Hams F-12 and three parts Liebowitz L-15 modified with amphibian salts and supplemented with toad serum, calf serum and beef embryo extract. 40% of primary cultures grow to confluency and are subcultured. 30% of lines subcultured continue to grow. Currently six lines of toad bladder epithelial cells are in continuous culture. One of these lines has been characterized with respect to transport (37).

Convincing evidence has been accumulated that the cells in culture retain characteristics of hormone sensitive transport typical of their tissue of origin (38). When grown on Nucleopore filters and studied in Ussing-type chambers, the cells develop a transepithelial electrical potential (P.D.) of 20.9 $\pm$ 2.6 mV, apical surface negative. The transepithelial resistance (r) is 10,9000 $\pm$ 100 ohm/cm^2. Both P.D. and SCC are markedly inhibited and r is increased by removal of all mucosal sodium or by exposure of basal surfaces of the cells to ouabain. These observations suggest that the SCC is primarily related to active sodium transport as it is in intact bladder. The cells in culture demonstrate marked stimulation of P.D. and SCC and decrease in r when exposed to aldosterone. Exposure to potent analogs of cyclic AMP causes a prompt increase in SCC primarily through a decrease in r. Amiloride appears to selectively block the aldosterone induced increment in SCC and has no effect on basal or C-AMP stimulated SCC. These results suggest that primary cell lines can be developed in culture for the detailed study of specific aspects of hormone sensitive transport.

Primary cell lines of epithelial cells from turtle urinary bladder, a tissue extensively studied in this laboratory, have been grown using similar techniques, but have not as yet developed into continuous lines capable of characterization with regard to their transport properties.

b. Mammalian kidney cells.

The cell line LLC-Pk_1, developed by Eli Lilly Co. is derived from an unknown cell type in pig kidney. This line has been shown to possess hormone sensitive adenylate cyclase activity (39) and may be a useful model system if it could be characterized with respect to its transport properties. Preliminary observations employing techniques developed for the study of toad urinary bladder cells indicate that these cells form an epithelial sheet in culture with a comparatively low potential difference (2 mv) apical surface negative, and a low transepithelial potential difference typical of "leaky" epithelia. Attempts will be made to determine the source of the potential difference in these cells and demonstrate whether it is sensitive to hormonal influences.

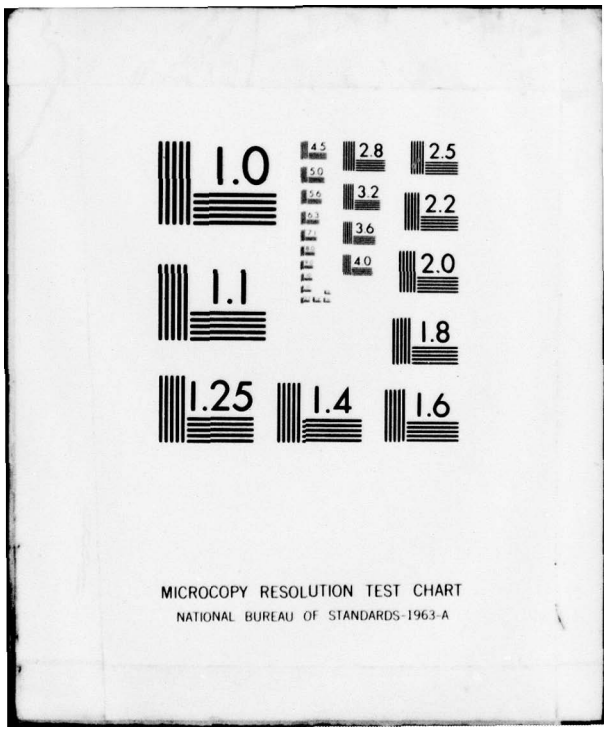
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Project 3M161102BS01 RESEARCH OF MILITARY INJURY AND DISEASES

Task 00

Work Unit 141 Pathogensis of Renal Disease of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOB 6532	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	142
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pathophysiology of Systemic Responses to Shock and Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING 78	3	143
FISCAL YEAR CURRENT 79	2	113

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Shelf Graft; (U) Vascular Trauma; (U) Umbilical Vein Graft; (U) Bovine Grafts; (U) Platelet Inhibition; (U) Sympathectomy

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To determine if platelet inhibitory drugs will improve the patency rate of human umbilical vein grafts and Bovine grafts used in arterial and venous repairs respectively. To determine if sympathectomy in combination with inhibition of platelet function will improve patency rate of Bovine grafts. These studies are militarily relevant in that they were designed to help develop a shelf graft for use in repair of both arterial and venous injuries which occur frequently in combat casualties.

24(U) Four CM segments of human umbilical veins or saphenous veins preserved with either gluteraldehyde or dialdehyde starch were anastomosed end to end to the transected ends of both ulnar and both femoral arteries in a canine model. Similarly, segments of Bovine grafts were anastomosed end to end to the transected femoral veins. A sympathectomy was performed and/or platelet inhibitory drugs were used.

25(U) 77 10-78 09 Twenty-five human vein grafts were studied from 3 days to 6 months after implantation. Only two of the grafts stayed patent. In 10 dogs receiving Bovine grafts and treatment with aspirin plus persantin, 4 grafts were patent at 14 days. Sympathectomy did not improve the patency rate in the 8 grafts which have been implanted. In 10 dogs receiving Bovine grafts and no treatment, none of the grafts were patent at 14 days.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3M161102B[illegible] RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 142 Pathophysiology of Systemic Responses to Shock and Trauma

Investigators
Principal: LTC Arthur W. Fleming, MC
Associate: LTC George J. Collins, MC
COL Norman M. Rich, MC

I. Effects of Aspirin on Dialdehyde Starch Treated and Glutaraldehyde Treated Human Umbilical Veins Used in Small Arterial Bypass Procedures

A. Background and Statement of the Problem. Many vascular substitutes have been proposed for repairing or replacing diseased or injured arteries or veins. These include autografts, allografts, zenografts, and synthetics. However, surgery on arteries measuring less than 2 mm in diameter is complicated by an unacceptable rate of thrombosis at the present time when any material other than autogenous vein or artery is used. Since autogenous material is not always readily available for vascular reconstructive procedures, especially in battle casualties, we have been searching for viable alternatives. The potential of using the modified umbilical vein prosthesis as an arterial substitute in a wide variety of vascular reconstructions has been pointed out by Dardik et al.[1] The promising advantages of umbilical veins are: they are readily available as an onshelf product, they handle and suture easily, they possess a biocompatible blood interface, they maintain structural integrity both mechanically and histologically, they are not immunologically rejected and they have no evidence of biodegradation. These vessels are also impervious, valveless, unbranched, and uniform in diameter.

B. Experimental Approach. Each dog was anesthetized with sodium thiopental (12.5 mg per kg), intubated and ventilated by a positive pressure respirator using room air. The ulnar artery on both sides was exposed. Heparin was administered proximally in the ulnar artery in a dose of 10 units per kg. A graft 4 cm long was then anastomosed end to end to the ulnar artery. A dialdehyde starch treated graft was placed on one side, and a gluteraldehyde treated graft was placed on the opposite side. At a later date, a similar procedure was carried out on the common femoral arteries. Following the completion of the anastomoses, electromagnetic flow measurements were made. Graft patency was further confirmed by arteriograms. Dogs were randomly placed in one of two groups. Those receiving no aspirin were placed in Group I and those receiving 1.2 gm of aspirin each day were placed in Group II.

C. Results and Discussion. Twenty-five human umbilical vein grafts were studied from three days to six months after implantation. All of the grafts placed in animals not receiving aspirin thrombosed. Only two of the grafts stayed patent in animals receiving aspirin. It should be noted that the size of the human umbilical veins were 3.1 and 4.0 mm in diameter respectively for the different manufacturers. However, the diameter of the vessels in which the anastomoses were carried out was usually only approximately 2 mm in diameter. Despite early patency as verified by electromagnetic flowmeter measurements at the time of operation, the majority of these grafts thrombosed. The measurement of platelet aggregometry using ADP, collagen, and epinephrine suggested that adequate levels of aspirin were present.

D. Conclusions and Recommendations. The initial trial with human umbilical veins was very disappointing. Our findings are in marked contrast to certain clinical studies that have been reported. Since we attempted to use very small vessels for anastomosis, we have now elected to focus our attention on the human umbilical artery which is usually 1 to 2 mm in diameter and more closely approximates the size of the vessel that we are attempting to repair.

II. Physiologic and Biochemical Tolerance to Multiple Phlebotomies

A. Background and Statement of the Problem. The quantitation of iron intake is deemed essential in interpreting the maximum volume of blood that can be safely donated. Recent clinical studies suggested that the normal adult male had decreased iron stores after the donation of 4 units of blood per year. As a consequence, current standards are that an adult can only donate 4 units of blood per year. However, the study on which this decision was made did not document the amount of iron intake. The physiologic and biochemical tolerance to frequent donations of blood were carried out in a canine model in which the precise amounts of iron administered could be quantitated.

B. Experimental Approach. Forty-six dogs weighing 19-34 kg were bled 12.5% of their estimated blood volume (EBV) on each of eight occasions over a period of 17 days. Blood samples were obtained prior to each phlebotomy for hematocrit (Hct), hemoglobin (Hgb), serum iron (SI), total iron binding capacity (TIBC), serum protein (SP) and serum electrolytes (SE). Dogs were randomly divided into 4 groups, each receiving 1 mg of iron per ml of blood withdrawn: (1) received oral iron only; (2) received multiple iron dextran injections intravenously (IV); (3) received a single bolus dose of iron IV; (4) received multiple iron injections intramuscularly.

C. Results and Discussion. Following the removal of 88 ml per kg of body weight, dogs receiving only oral iron had a decrease in the Hct from 44.3 ± 1.3% to 36.5 ± 1.3% and a decrease in the Hgb from 16.1 ± 0.6 grams percent to 12.8 ± 0.7 grams percent. Erythropoiesis as manifested by the Hct and Hgb was not further enhanced by the addition of parenteral iron ($p<.05$). SI, TIBC, and SE were not significantly altered ($p<.05$). SP decreased from 7.7 ± 0.4 to 6.3 ± 0.1 grams percent, however, there was only a minimal change in the serum albumin ($p<.05$). There were no episodes of arterial hypotension or change in the arterial-venous difference in oxygen content, after undergoing a general anesthetic and thoracotomy 4 days after the last phlebotomy.

D. Conclusions and Recommendations. With a documented adequate daily intake of iron, approximately 100% of the estimated blood volume can be removed in a period of 17 days without detrimental effects in a dog. This suggests that healthy blood donors can donate more frequently if adequate exogenous iron is taken.

III. Development of a Practical Autologous Blood Transfusion Program

A. Background and Statement of the Problem. The risks associated with the use of homologous blood transfusions are well documented and include significant acute problems, as well as long-term morbidity and/or mortality (allergic and febrile reactions, viral hepatitis and other infectious diseases, Rh sensitization and non-red cell antigen sensitization). The exclusive use of autologous blood transfusions (autotransfusions) for elective operations would eliminate almost all of the disadvantages of homologous blood transfusions. In addition, many of the logistical problems in obtaining certain blood types would be decreased, the tremendous drain that is occasionally made on blood bank stores would be greatly reduced, and national blood resources could be conserved. In addition to wishing to develop a self-perpetuating system, we also wanted to provide a model for other surgical subspecialties.

B. Experimental Approach. Patients who were to undergo elective surgical procedures at Walter Reed Army Medical Center (WRAMC) were interviewed by a member of the surgery team. The need for transfusions and the safety inherent in using autologous blood were explained in detail. If the patient was accepted into the program, an informed consent form had to be signed by the patient or his/her legal guardian. Blood was then donated and collected according to American Association of Blood Bank Standards and preserved as full blood, fresh frozen plasma, packed cells or frozen packed cells.

C. Results and Discussion. Three hundred and six patients donated one or more units of autologous blood before undergoing an elective surgical procedure at Walter Reed Army Medical Center. Although some of the versatility and flexibility of our program could be directly attributed to the use of frozen blood, lacking this capability should not deter surgical services from being involved in an active autologous blood transfusion program. The number of units of blood most frequently requested and used for elective operations in our Institution (excluding open heart surgery) was two units.

D. Conclusions and Recommendations. The enthusiasm which patients with severe cardiovascular disease have shown for this program, the minimal problems that have been encountered, the freedom from severe complications, the multiple benefits derive and the subsequent involvement of those in other surgical subspecialties suggest that this program can grow in scope and depth as time passes. Although we strongly endorse the use of autologous blood for all truly elective operations, there can be no substitute for the overall judgement of the physician especially for patients with coronary artery disease.

IV. Demonstration and Removal of Cardiotoxic Substances in Irreversible Hemorrhagic Shock

A. Background and Statement of the Problem. The main cause of death for surgical patients in Vietnam between 1 January - 31 December 1969, other than head injuries (42.5%), was hemorrhagic shock which occurred in 23.9% of patients.[2] The term hemorrhagic shock was used to describe the cause of death when the patient developed cardio-respiratory arrest following an episode of peripheral vascular collapse which was considered to be directly related to massive bleeding not responsive to blood transfusions. Wiggers in 1945 reported results that strongly suggested that failure of transfusions in hemorrhagic shock was not necessarily due to unsuitability of solutions used; nor chiefly to loss of plasma or solutions through damaged capillaries; or failure of venous return; but, frequently at least, was attributable to myocardial depression, i.e., to a reduced reaction to equivalent venous filling pressures.[3] Our hypotheses was that these cardiotoxic substances can be removed from the circulation by total body washout.

B. Experimental Approach. Animals were bled approximately 100% of their estimated blood volume over a period of 17 days to obtain blood for reinfusion following hemorrhagic shock. Animals were placed in hemorrhagic shock using a modified Wiggers technique. Animals were maintained at a reduced arterial pressure of 50 mm of

mercury for 90 minutes and then at a critical level of 30 mm of mercury for another 45 minutes by careful withdrawal of small additional amounts of blood. At the time of resuscitation, a total body washout was performed using a clear prime and total cardiopulmonary bypass. The banked blood that had previously been drawn was then slowly reinfused and diuretics administered to bring the hematocrit back to normal.

C. Results and Discussion. Preliminary data has demonstrated that this modified Wiggers model is uniformly fatal in the control group. The technique of total body washout and reinfusion of autologous banked blood is still in its preliminary stages, however, results to this date have been promising. Initial problems have been with a marked degree of hemodilution. The recent acquisition of a cell-saver is expected to help reduce the degree of hemodilution that has been encountered thus far.

D. Conclusions and Recommendations. Total body washout and autologous banked blood still holds promise as a potential technique for reversing irreversible shock. At the present time, this technique is considered as still in the developmental stage.

V. Vena Caval Interruption in Pulmonary Hypertensive Canines

A. Background and Statement of the Problem. In 1934, Homan's recommended venous interruption for the prevention of pulmonary embolism. Since that time, many prominent surgeons have recommended either partial or complete interruption of the inferior vena cava for the prevention of pulmonary embolism. Physiologic studies comparing the impact of these two procedures, i.e., partial or complete inferior vena caval interruption have been gathered in normotensive rather than pulmonary hypertensive animals. In these experimental preparations, it has not been possible to conclusively demonstrate whether or not partial interruption has a lesser physiologic impact than complete interruption. The purpose of our study was to measure the physiologic effects of partial and complete vena caval interruption in pulmonary hypertensive animals.

B. Experimental Approach. Thirty adult mongrel dogs of either sex weighing between 20 and 30 kgms were anesthetized with pentobarbital (30 mgm/kgm). A large bore cannula was inserted into the left external jugular vein. A Swan-Ganz catheter was inserted into the pulmonary artery via the right external jugular vein. The right femoral artery and vein were cannulated with polyethylene tubing. Measurements taken during each experiment were systemic arterial pressure, pulmonary arterial pressure, femoral venous pressure, cardiac output, and heart rate. In addition, arterial pO_2, pCO_2, pH, saturation and hemoglobin concentration were determined.

Animals were divided evenly into three groups on a random basis: Group I animals had pulmonary embolisms only; Group II had pulmonary embolisms plus inferior vena caval ligations; and Group III animals had pulmonary embolisms plus inferior vena caval clippings using a four channel serrated Teflon clip. Serial determinations of the previously mentioned parameters were made at 15 minute intervals for two hours. Chest roentgenograms and inferior vena cavagrams were obtained in five dogs from the group having pulmonary embolisms plus inferior vena caval clippings and five dogs from the group having inferior vena caval ligations.

C. Results and Discussion. The objective of effecting a 20-25% reduction in systemic arterial pressure with stabilization was achieved in 93.3% of dogs. The pulmonary embolism sufficient to cause the desired level of hypotension regularly caused increased pulmonary arterial pressure. Right heart strain, as reflected by an increase in femoral venous pressure, also occurred in all groups. Despite differences in absolute numerical values, the increase from baseline to post-embolic femoral venous pressure was statistically significant in all groups. Cardiac output response to embolism was variable but was reduced after embolism in 63.3% of dogs. Heart rate response was also variable with only 33.3% of dogs having an increased heart rate when first measured after massive pulmonary embolism. Throughout the two hour observation period, cardiac output continued to fall in all groups. Both vena caval clipping and ligation caused a lowering of cardiac output over and above that which occurred with pulmonary embolism alone. By the end of the observation period, cardiac outputs were essentially equal for both types of caval interruption but both of these groups of animals had lower outputs than dogs having pulmonary embolism alone. Although initial cardiac output responses were variable, by the end of the two hour observation period all dogs had lower cardiac outputs than before embolism. In all groups, systemic arterial pressures remained below pre-embolism levels throughout the two hour observation period. In the group undergoing vena caval ligation, there was a fall in systemic arterial pressure from 147.5 $\pm$ 12.2 before embolism to 111.9 $\pm$ 8.3 after embolism. This was followed by a secondary fall to 92.5 $\pm$ 4.7 measured fifteen minutes after ligation ($p<0.025$). In the group undergoing partial vena caval interruption, there was only a slight fall from 104.7 $\pm$ 3.5 to 100.0 $\pm$ 7.2 when measured fifteen minutes after clipping. This was not statistically significant. After embolism, pulmonary arterial pressure remained above pre-embolism levels in all groups. Similarly, femoral venous pressure remained above pre-embolism levels throughout the two hour observation period in all groups. The value after two hours of 22.4 $\pm$ 2.3 in the ligation groups was significantly higher than either the value of 7.7 $\pm$ 2.1 in the group of pulmonary embolism plus vena

caval ligation.

These data demonstrate that massive pulmonary embolism causes severe hemodynamic derangements. While minor pulmonary emboli may cause insignificant and often transitory changes in parameters such as systemic arterial pressure, pulmonary arterial pressure, and cardiac output, repetitive emboli have an additive effect and a point will eventually be reached at which a sustained elevation of pulmonary arterial pressure occurs. These relationships have been previously demonstrated by us when successive five cc volumes of autologous clot were embolized and pulmonary arterial pressures were measured. Concurrent with the increase in pulmonary arterial pressure one may expect a decrease in systemic arterial pressure. If the embolus is massive enough to produce right heart strain, peripheral venous pressure will also become elevated. On a purely mechanical basis, a truly massive embolus must occur in order to effect these hemodynamic derangements. The actual weight of glass beads that were embolized in our experiment averaged 73.1 gms.

Our results indicate that pulmonary embolism itself causes and may sustain severe hemodynamic derangements. Either method of vena caval interruption then has a significant impact in animals with impaired cardiovascular homeostasis. Our data argue only slightly in favor of a lesser impact from vena caval clipping than from vena caval ligation. Vena caval ligation caused a greater decrease of cardiac output and systemic arterial pressure at 15 minutes than vena caval clipping. Presumably this was because of an acute reduction of venous return, but definite conclusions should not be drawn since the differences between groups were not statistically significant. In the face of cardiac decompensation, vena caval ligation could presumably be beneficial because of a phlebotomy effect. Any such effect, may be short lived because of the rapid development of venous collateral channels. We were able to demonstrate the rapid opening of such collateral channels by doing phlebograms in the immediate post-ligation period.

D. Conclusions and Recommendations. It can be concluded from these studies that when either vena caval clipping or vena caval ligation are performed in the face of altered hemodynamics as in the unstable individual who has undergone massive pulmonary embolism, there will be a further reduction of systemic arterial pressure and cardiac output. After a period of 90 to 120 minutes, the effects of these two procedures appeared to be equivalent. When sufficient venous collaterals are present, the major determinant of the ultimate outcome is the impact of the embolus itself rather than the type of procedure used to interrupt the inferior vena cava. Because of the inability to predict the presence or absence of such collaterals in the operating room or even preoperatively, we are swayed by these experiments to recommend

partial rather than complete interruption of the inferior vena cava for prevention of pulmonary embolism.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 142 Pathophysiology of Systemic Responses to Shock and Trauma

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6467	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	143
b. CONTRIBUTING				
c. ~~XXXXXXXX~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Gastrointestinal Responses to Shock and Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

008800 Life Support 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	2	120
	CURRENT 79	2	137

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: Harmon, MAJ, John
NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Gastric Mucosal Barrier; (U) Abdominal Visceral Blood Flow; (U) Septic Shock; (U) Portacaval Shunt

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To evaluate the effects of septic shock on the integrity of the gastric mucosa and upon gastrointestinal visceral blood flow. To determine the effect of gram-negative bacterial infection as opposed to pharmacologically prepared "endotoxin." To determine the effects of various portacaval shunts on gastrointestinal visceral blood flow. War combat casualties with abdominal injuries frequently develop gram-negative bacterial infection and occasionally receive injuries requiring portacaval type shunts.
24 (U) Radioactively tagged microspheres were used for flow measurements. These were injected into the heart where they mix with the blood. Microspheres were then distributed to the periphery with the blood. A reference sample was taken at a known flow rate to determine actual flows. Intraabdominal sepsis in Rhesus monkeys was produced by injecting E-Coli bacteria into the gall bladder in which the cystic duct and artery had been ligated.
25 (U) 77 10 - 78 09 The model for abdominal sepsis is being developed. Difficulty was encountered determining the appropriate dose of E-Coli to produce sepsis without producing death in the first 24 hours. The microsphere techniques for determining blood flow are now well developed. Methodologic problems for evaluating the primate gastric mucosal barrier still need resolution. Evaluation of the effect of portacaval shunts with microspheres is ongoing. Hepatic arterial blood is increased by approximately 100 percent one hour after side to side portacaval or interposition mesocaval shunts. Flows return to control values by 3 weeks after shunting, however.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 143 Gastrointestinal responses to shock and trauma

Investigators
Principal: MAJ Clayton H. Shatney, MC
Associate: MAJ John W. Harmon, MC

I. Comparison of Total Hepatic Arterial Blood Flow using Gamma-Labeled Microspheres Following Porta-systemic Shunting

A. Background. Porta-caval shunts have been extensively utilized in the treatment of bleeding esophageal varices secondary to hepatic cirrhosis. This procedure also has a potential role in the treatment of intestinal schistosomiasis and in the reconstruction of the traumatized portal system.[1] Although portacaval shunts are effective in controlling variceal hemorrhage, hepatic failure following shunting can occur acutely or chronically and appears in a significant percentage of shunted patients.[2] Hepatic decompensation has been attributed to the loss of portal inflow to the liver by the shunt. Because of this possibility the interposition (H graft) mesocaval shunt was developed, and its proponents claimed that it was hemodynamically superior to the standard porta-caval shunts.[3]

This laboratory recently studied the portal hemodynamics of three portasystemic shunts using gamma-labeled microspheres to evaluate portal blood flow.[4] A comparision was made between the hepatopetal portal blood flow following end-to-side and side-to-side porta-caval shunts and mesocaval interposition shunts. The microsphere technique showed that about 60% of the portal flow to the liver was preserved by the mesocaval shunt and virtually none by the side-to-side porta-caval shunt. The results indicate, therefore, that the side-to-side porta-caval shunt is identical to the end-to-side shunt with regards to portal hemodynamics. Since the incidence of hepatic failure is reportedly less after side-to-side porta-caval shunting, changes in portal blood flow cannot be solely responsible for hepatic decompensation after portasystemic shunts.

Another possible contributing factor in post-shunt hepatic failure is the volume of arterial blood flowing to the liver. It has been postulated that with the loss of portal inflow there is a compensatory increase in hepatic arterial blood flow in order to provide sufficient oxygen to the liver for its metabolic needs. However, there is no general agreement in the literature on this point.[5,6] Some studies have shown increased arterial flow, while others have demonstrated a reduction in both hepatic

arterial blood flow and oxygen consumption following porta-caval shunting.[7,8] We attempted to resolve the question of arterial blood flow to the liver after portasystemic shunts with the use of gamma-labeled microspheres, which allowed us to determine the total input to the liver from all arterial sources.

B. Experimental approach. Fifteen mongrel dogs anesthetized with Halothane were randomly assigned to three groups: sham operated, side-to-side portacaval shunt, and H-graft mesocaval shunt. Pulmonary artery pressure, cardiac output, hepatic arterial blood flow and visceral blood flow were determined prior to and 1 hour and 3 weeks after shunting, when the animals were sacrificed. Cardiac output was measured by thermodilution and organ blood flow with 50±3 u radioactive microspheres (^{51}Cr, ^{85}Sr, ^{141}Ce) injected into the left ventricle.

C. Results and discussion. Cardiac output and pulmonary artery pressure did not change significantly during the study. Hepatic arterial blood flow (HABF) and individual visceral organ blood flow (ml/100 gms tissue/min) are tabulated below (mean flow ±SE).

	Liver (HABF)	Small Bowel	Large Bowel	Spleen	Pancreas	Stomach	Omentum
Sham							
Baseline	19±4	38±6	50±8	123±28	31±8	52±12	6±2
1 hour	33±4	45±11	47±9	124±34	40±15	31±8	19±3*
3 weeks	23±10	42±8	38±6	139±31	46±15	30±5	5±1
Mesocaval							
Baseline	29±10	29±7	58±12	86±24	47±12	35±9	2±1
1 hour	81±9*	36±8	49±7	119±46	48±16	51±16	19±6*
3 weeks	38±8	75±14*	60±13	135±36	63±26	39±3	6±1*
Portacaval							
baseline	17±3	22±2	37±5	172±25	56±18	28±7	2±1
1 hour	46±10*	41±6*	52±8	134±37	87±26	40±6	17±4*
3 weeks	24±8	48±8*	47±10	86±8*	86±25	35±10	4±2

*differs from baseline at $p < .05$

Total hepatic arterial blood flow significantly increases immediately after mesocaval or side-to-side portacaval shunting, but within 3 weeks hepatic arterial blood flow returns to pre-shunt levels. The increase in HABF is independent of changes in cardiac output or circulating blood volume, as gauged by pulmonary artery pressure, and is apparently a transient compensatory response to the reduction in hepatic portal flow. The persistent increase in small bowel blood flow after both shunts and the changes in splenic and omental flow after

portacaval and mesocaval shunting, respectively, probably reflect changes in outflow resistances, which vary for each visceral organ with the type of shunt.

D. Conclusions and recommendations. Following either side-to-side portacaval or H-graft mesocaval shunting there is a transient, significant increase in hepatic arterial blood flow and a long-term increase in blood flow to the small intestine. Our data indicate the need to evaluate distal portal vein arterialization in conjunction with portasystemic shunting as a means of preventing the permanent loss of much of the portal hepatic blood flow after shunting — a loss for which the hepatic arterial circulation does not compensate.

II. Changes in the Integrity and Function of Primate Gastric Mucosa During Gram-negative Septic Shock. Development of a Model System

A. Background. Gram-negative septic shock remains a serious threat in our hospitals, despite a large body of knowledge gained through clinical and experimental investigations. The incidence of gram-negative-rod bacteremia has steadily increased during the past two decades through the combination of a number of host, agent and transmission factors.[9,10] Renal failure, respiratory insufficiency, heart failure, and gastrointestinal bleeding are frequent complications of septic shock. Approximately 30% of these patients experience at least one episode of upper gastrointestinal bleeding, which is due to hemorrhagic (stress) gastritis in the vast majority of cases.[11]

Septic shock and its complications have been extensively investigated during the past 20 years. Endotoxin shock has been the primary method of studying the pathophysiology and treatment of septic shock. However, due to numerous pathophysiologic differences between human septic shock and experimental endotoxin shock, the latter has frequently been criticized as being an inadequate reproduction of the clinical situation.[12,13]

The need for an experimental preparation of septic shock which closely approximates the clinical setting prompted a series of investigations. A new model of septic shock was developed, based on the creation of an ischemic, infected (E. coli) gallbladder in dogs.[14] As in clinical septic shock, two distinct patterns of hemodynamic responses were observed. About 25% of the animals developed a normo- to hypodynamic circulatory status, and 75% responded with an increase in cardiovascular dynamics. The mortality rate was 100% in the low flow group, with a mean

survival of three days. The high flow subset experience a mortality rate of 90% with a mean survival of five days. Virtually all animals had bloody diarrhea following the introduction of sepsis. We proposed to use this same technique in the Rhesus monkey to see if we could develop a new model to study the effects of sepsis and shock on the integrity and function of the gastric mucosa.

B. Experimental approach. Rhesus monkeys weighing 4-8kg were tranquilized (2mg/kg Sernylan IM) and brought to the operating room. Prior to anesthesia gastric mucosal integrity and potential difference (P.D.) were measured endoscopically using trans-endoscopic electrodes. The monkeys were then anesthetized with Halothane. Arterial and venous cannulas were introduced, as well as Swan-Ganz catheters. After baseline hemodynamic and hematologic studies, the abdomen was opened and the cystic artery and duct ligated. A suspension of E. coli (Type ATCC 25922) containing 10^8, 10^6 or 10^4 organisms per kg was injected into the gall bladder. The abdomen was then closed, and the animal returned to his cage. Thereafter gastric, hemodynamic and hematologic studies were repeated daily until the monkey succumbs from septic shock.

C. Results and discussion. Initial investigations have indicated that a dosage of E. coli containing 10^8 and 10^6 organisms per kg in the monkey leads to death in 24 hours and 42 hours respectively. These investigations have demonstrated the feasibility of this technique for the production of septic shock in the Rhesus monkey. However, a dosage of 10^4 E. coli per kg appears to be ideal for this species, since it is perferable that the animals remain septic for approximately 2-4 days before succumbing.

D. Conclusions and recommendations. Preliminary studies have demonstrated the feasibility of using an infected, ischemic gall bladder model to create septic shock in the Rhesus monkey. An innoculate of 10^4 E. coli per kg appears to be the most adequate dosage. Formal investigation of this new septic shock technique is currently being pursued.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 143 Gastrointestinal response to shock and trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6527	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	144
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Control Mechanisms of Regional Circulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	D. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	3	135
FISCAL YEAR CURRENT 79	2	133

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Oxygen Comsumption; (U) Tissue Respiration; (U) Organ Perfusion; (U) Pulmonary Function; (U) Sickle Cell Disease; (U) Cutaneous Blood Flow

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To refine techniques for quantifying regional blood flow and to define the role of the central nervous system on the control of cardiovascular responses. To determine whether sickle hemoglobin red cells treated with pyridoxine can be altered to have a reduced affinity for oxygen and thus an increased delivery of oxygen to tissues. To determine the airway closing capacity during general anesthesia. Further, to define the characteristics of cutaneous blood flow after wound production. These studies apply directly to respiratory and hemodynamic support of soldiers with acute battle injuries and/or post surgical stress.

24 (U) In vivo and in vitro physiologic techniques have been employed. Five anesthetic agents were tested. Three different radioactively tagged microspheres were used to measure the distribution of blood flow.

25 (U) 77 10 - 78 09 A thermal diffusion probe has been developed which can be used to monitor perfusion in small volumes of skeletal muscle. By using multiple probes, regional variation in a single muscle bed can be monitored continuously. The presence of thermally significant (large) vessels can be detected and corrected for. Pyridoxine and Pyridoxal phosphate have been found to inhibit sickling with a concomitant left shift of the oxygen-hemoglobin dissociation curve. After specific skin wound production, blood flow reached maximal levels after one week, suggesting that this is the preferred time for delaying surgical skin flaps for covering wound defects. Cardio-augmentation was elicited by electrical stimulation of the thoracolumbar spinal cords and showed strong right sided preponderance.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 144 Control mechanisms of regional circulation

Investigators
Principal: MAJ Alexander M. Guba, Jr., MC
Associate: MAJ Monty Woods, MC
MAJ William Reynolds, MC

I. Regional Hemodynamics of a Pedicle Flap

A. Background and Statement of the Problem. Arterial blood flow to skin flaps has not been serially, quantitatively measured. Experiments were performed in the dog. Blood flow was measured in each animal at three times. Both total flow and the distribution of flow were studied.

B. Experimental Approach. Using radioactively tagged microspheres under controlled conditions, blood flow was determined in island pedicle skin flaps prior to any surgical manipulation, 30 minutes after completion of raising the skin flap and 90 minutes after flap completion.

C. Results and Discussion. Blood flow uniformly increased in each skin flap studied ($p < 0.05$). This increase persisted 90 minutes postoperatively. Blood flow was uniform along the length of the flap; however, blood flow decreased with increasing distance from the midline.

These studies document the ability of microsphere injection to reproducibly measure cutaneous blood flow. In addition, the complexity of blood flow in a viable flap has been demonstrated.

II. Regional Hemodynamics in Delayed Skin Flaps.

A. Background and Statement of Problem. The physiologic alternations occuring with a surgical delay in skin flaps have not been quantitated. Using a pig model, flaps were designed and blood flow to the skin flaps was determined.

B. Experimental Approach. Temperature, blood gases, cardiac output were measured. Blood flow was then measured using 15 micron radioactively tagged microspheres at three times; prior to any surgical manipulation, after increasing periods of delay (2 days to 14 days), and 24 hours after final raising of the skin flaps.

C. Results and Discussion. Blood flow in the delayed skin flaps increased with increasing delay intervals, reaching maximal levels after one week, $p < 0.05$. This increase paralleled an increase in surviving flap length.

These studies quantitate one important physiologic aspect of the delay phenomenon: an increase in nutrient capillary flow during the delay period.

III. Coverage of Exposed Vascular Conduits

A. Background and Statement of the Problem. Exposed vascular bypass grafts subject the surgical patient to infection and life threatening hemorrhage. Infected grafts must be replaced. However, on occasion, exposed grafts can be covered by local surgical techniques.

B. Experimental Approach. The records of patients with exposed vascular grafts in the Vascular Clinic at WRAMC were located. Their treatment was reviewed.

C. Results and Discussion. Two patients were successfully treated with local surgical techniques. In one patient, an exposed saphenous vein bypass graft was covered with a split thickness skin graft; in another patient, an exposed axillo-femoral bypass graft was covered by a rotational flap.

These procedures have broadened the surgical armamentarium available to surgeons treating these difficult patients.

IV. Vascular Access: Additional Therapeutic Utilization.

A. Background and Statement of the Problem. Vascular access procedures have been performed to allow renal dialysis in renal failure patients. Such procedures can also be useful in patients requiring multiple transfusions, chemotherapy, and treatment of diabetic ketoacidosis.

B. Experimental Approach. Records were reviewed in the Vascular Clinic in WRAMC of patients who had undergone vascular access procedures for non-dialysis purposes.

C. Results and Discussion. Thirteen patients underwent these procedures and successfully obtained medical therapy via these access procedures.

This study emphasizes the utility of vascular access in many medical diseases. In addition, the variety of surgical technical options is presented.

V. A Microprocessor Based Thermal Diffusion Probe for Blood Flow Measurements.

A. Background and Statement of the Problem. Attempts at measuring tissue perfusion with the aid of thermal devises has met with limited success in the past. Over the past four years, experiments have been performed in our laboratory in an effort to develop a thermistor probe to measure tissue perfusion. These experiments have been carried out in collaboration with engineers at MIT and Northeastern University with the aim of modifying the technique for direct measure of tissue blood flow.

B. Experimental Approach. The purpose of these experiments was to evaluate instrument and probe function in the continuous mode. Multiple probes were inserted into an isolated gracilis muscle preparation. Muscle flow was varied and flow was computed simultaneously and continuously.

C. Results and Discussion. The local fluctuation in gracilis flow determined during continuous mode operation were of the same magnitude as the scatter observed when flows measured by transient mode operation and collected venous return were compared. Further experimentation will be required to determine how much of this scatter is experimental error, and how much represents normal physiologic behavior.

VI. Studies of Airway Closure During General Anesthesia.

A. Background and Statement of the Problem. Recent studies have indicated that reduction in functional residual capacity (FRC) with resultant airway closure is a major factor in the development of hypoxemia during general anesthesia. Specifically, the relationship between FRC and the closing capacity (CC) is believed to underlie the increase in venous admixture seen in anesthetized patients. Since various anesthetic agents are known to affect the tone and stability of the airways, this implies that the FRC-CC relationship, and consequently the magnitude of venous admixture, may vary among anesthetic agents and techniques.

B. Experimental Approach Adult baboons were used. Anesthesia was induced with a deep intramuscular injection of methohexital in a dose of 6mg/kg. Animals were then

paralyzed with succinyl choline, intubated, and ventilated until return of spontaneous ventilation. Animals were then connected to an anesthesia circuit and general anesthesia maintained with one of the following agents: (a) Halothane; (b) Enflurane; (c) Fluroxene; (d) Ketamine; or (e) Fentanyl. The FRC was calculated from helium dilution data and the CC was measured using a variation of the foreign gas bolus method.

C. Results and Discussion. All of the anesthetic agents used caused a reduction in FRC and resulted in some degree of airway closure. The reduction in FRC could however be reversed by positive end expiratory pressure.

D. Conclusions and Recommendations. Having developed and validated the technique for measuring airway closure with different anesthetics, studies need to be carried out under the following conditions: (1) Mechanical versus spontaneous ventilation; (2) Different postures (prone, sitting, and lateral decubitus); and (3) After administration of aersolized histamine in the inspired gas mixture (both with and without antihistamine pre-medication).

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 144 Control mechanisms of regional circulation

Literature cited.

Publications:

1. Guba, A.M., Zinner, M., Hobson, R.: Regional hemodynamics of a pedicle flap. J. Surg. Res. In press.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6458	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	145
b. CONTRIBUTING				
XXXXXXXXXX	Cards 114F			

11. TITLE (Precede with Security Classification Code): (U) The Effects of Specific Environmental Pollutants on the Biosynthetic Functions of Mammalian Cells in vitro; A search for Structure/Activity Relationships

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
002600 Biology 005900 Environmental Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 08	Cont	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	67
	CURRENT 79	1.0	90

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Environmental Toxicology; (U) In Vitro Toxicology, (U) Munitions Pollution

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To develop in vitro systems for toxicology screening of polluting products of the manufacture of munitions, to determine the mechanism of action of the toxic effect, and to find structure-activity relationships with predictive value. By executive order, the Army has been charged with complying with EPA regulations and policies, EPA requires toxicologic studies of manufacturing wastes to establish standards.

24. (U) Replicate in vitro hemoglobin synthesis including heme and globin synthesis, erythroblast formation and maturation. Compounds of interest (initially benzene, nitrobenzene and nitrotoluenes) to the Army will be introduced in vitro, and mode of action determined.

25. (U)77 10-79 09. In vitro systems for examination of heme synthesis (rat liver homogenates), globin synthesis (rabbit reticulocyte lysate), and erythroid cell proliferation (plasma clot culture of dog bone marrow) were established and characterized. Special efforts were made to standardize each of the systems as much as possible to insure comparability of results obtained at different times. Several vehicles for delivery of the benzene and toluene derivatives were compared. Comparative toxicity data were obtained for a limited number of compounds, at concentrations ranging from 0.000001 to 0.001, using all three test systems. The compounds examined in detail include: benzene, o-, m-, and p-dinitrobenzenes and 2,3; 2,4; 2,5; 2,6; 3,4; and 3,5 dinitrotoluenes. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

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PROJECT 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 145 The Effects of Specific Environmental Pollutants on the Biosynthetic Functions of Mammalian Cells In Vitro; A search for Structure/Activity Relationships

Investigators

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Description:

The basic goal of this research project is to determine whether relative in vitro toxicities of a group of compounds arising from breakdown of a particular pollutant can be predicted by (a) examining the relative in vitro toxicities of a small number of such derivative compounds with different structural characteristics, and (b) using structure-activity relationships to predict which others will be most toxic in the in vitro system. The specific test systems we are using are heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system, and erythroid cell proliferation in a plasma clot culture system.

The first year's work on this proposal concentrated on three areas: (a) establishing and characterizing the three principal test systems; (b) obtaining toxicity data for a limited number of compounds using all three test systems,and examining the relationships between data obtained for a given compound by each system; and (c) utilizing one of the systems, with which it is easier to perform a large number of dose-toxicity

studies, to screen a large number of nitrotoluenes and their analogues in order to determine which group of compounds may show significant structure/activity relationships. Progress in each of these three efforts is discussed below.

Progress:

A. Establishment and characterization of the three principal test systems

1. Heme synthesis

In this area, we have focused our attention for the past year on ALA synthetase and heme synthetase in rat liver homogenates. ALA synthetase was measured by a minor modification of the method of Ebert et al (1). Heme synthetase was measured by a modification of the method of Bonkowsky et al (2). In this case the modification was a major one; we used mesoporphyrin IX, rather than protoporphyrin IX. Because mesoporphyrin IX is much easier to solubilize, we were able to use much higher porphyrin concentrations than Bonkowsky et al had been able to achieve with protoporphyrin IX, and we were able to get much higher enzyme activity. Our description of this modification is currently being prepared for publication. Control values for liver ALAS were 1-3 nmol/g protein/0.5 hr and for HS they were 16-27 μ mol/g protein/hr. Each assay was characterized with regard to a number of variables: (a) linearity over time, (b) relation of activity to protein concentration in the homogenate, (c) reproducibility of the assay performed the same day on multiple aliquots of homogenate from the same liver, (d) stability of the enzyme when frozen as freshly prepared homogenate at -70°C and stored for several weeks, and (e) comparison of activity in homogenates from different animals. In addition, since our assay for heme synthetase involves a substantial modification of previously reported procedures, we have performed kinetic studies of this enzyme as well.

Linearity of ALA synthetase with respect to time is shown in Figure 1. The assay is linear for the first 30 minutes, and then begins to taper off; the decrease in ALA formed vs. time becomes marked beyond 45 minutes of incubation. Linearity of heme synthetase

activity with respect to time is shown in Figure 2. This assay is linear for the first 45 minutes, and then appears to become slightly faster. (The slightly lower than expected value at 60 minutes appears to be an artifact).

The relation of ALA synthetase activity and heme synthetase activity to homogenate protein concentration are shown in Figures 3 and 4 respectively. At non-limiting concentrations of alpha-ketoglutarate, the rate of the ALA synthetase reaction increases in a roughly linear manner with increase in protein concentration (Figure 3). Similarly, at non-limiting concentrations of ferrous iron and of mesoporphyrin, the rate of the heme synthetase reaction increases in a linear manner with increase in protein concentration up to 15.36 mg/ml (figure 4).

Within day reproducibility of ALA synthetase and heme synthetase determinations are shown in Tables I and II. The first column of Table I (Day 0) shows results of ten ALA synthetase assays performed the same day on aliquots of pooled rat liver homogenates. Table II shows results of three to eight heme synthetase assays performed on each of seven individual liver homogenates. In the case of both the pooled homogenate (Table I, Day 0) and the individual liver homogenates (Table II, Homogenate 1; Table II, Homogenate 2; etc), there was a high degree of within day reproducibility of assays on the same sample.

Stability of ALA synthetase and heme synthetase, when frozen at -70°C as freshly prepared homogenates and stored for up to several weeks, are shown in Tables I and III, respectively.

Significant variations in heme synthetase activity in homogenates prepared from different rat livers are demonstrated in Table II. Preliminary studies with ALA synthetase indicates that the same is true for that enzyme. It can be seen that variation from animal to animal is significant, and is certainly greater than that from day to day with frozen pooled homogenates.

Kinetic studies performed on heme synthetase are shown in Figures 5 and 6 and in Table IV. In Figure 5, the concentration of iron is varied, with the

concentration of mesoporphyrin remaining constant at 1000 nanomoles per reaction aliquot. Within the concentration range of iron tested, the enzyme activity increased in a nearly linear fashion with increased iron concentration. (There is a slight tapering at the highest iron concentration). In Figure 6, the concentration of mesoporphyrin is varied, with the concentration of iron remaining constant at 500 nanomoles per reaction aliquot. When activity (ordinate) is plotted against mesoporphyrin concentration (abscissa), the resulting curve is hyperbolic, but does not plateau even at 2650 nanomoles of mesoporphyrin, suggesting that the saturation level for mesoporphyrin may be even higher than 2650 nanomoles per aliquot of reaction mixture. In Table IV, a preliminary experiment showing the effect of added heme on heme synthetase activity is demonstrated. Although the results are not entirely clear-cut from this initial experiment, the general pattern seems to be one of enzyme inhibition at heme levels of 20 or more nanomoles per aliquot of reaction mixture.

2. Globin synthesis

Reticulocytes were obtained from New Zealand white rabbits after induction of reticulocytosis by phenylhydrazine injection, essentially as described by Crystal et al. (3). Rabbits were bled by cardiac puncture and the reticulocyte-enriched (75-90%) red blood cells collected by differential centrifugation and freed of plasma by washing in buffer. Membrane-free lysates prepared by this method can be stored at -85°C for at least one year with no significant loss in ability to synthetize globin.

Globin synthesis was determined by measuring the incorporation of ^{3}H-leucine into acid-insoluble protein. The procedures used were those described by Woodard et al. (4).

Although one need only add a radiolabeled amino acid to monitor the endogenous protein biosynthetic activity, the rate and extent of translation can be increased significantly by the addition of: (1) hemin, which blocks the formation of a translational inhibitor, and (2) an energy (ATP) generating system - in our case creatine kinase/creatine phsophate. The effects of adding other components required for translation i.e.,

amino acids, ATP, GTP, etc., were tested but no additional increase in activity was observed.

Figure 7 shows a time course for the incorporation of ^{3}H-leucine into globin protein using the supplemented lysate system from rabbit reticulocytes. Under the conditions of our assay the rate of amino acid incorporation is linear for about 40 minutes after which time there is a decrease in activity. While it is clear that the activity continues for at least an hour we have not measured incorporational activity beyond that time. Similar results have been obtained with four other preparations, although the relative activities vary from 50-120% of that shown (see Table V).

In a typical control assay, from 2-15 pico moles of leucine have been incorporated after 60 minutes of incubation. This level of activity is in good agreement with that reported by a number of other workers in the field. With regard to precision, analysis of quadruplicate data points from 21 independent assays performed with five independent preparations of lysate show the variance due to experimental error to be less than + 7-8%. When evaluating the effects of solvents and pollutants, incorporation measurements were made in duplicate at both 40 and 60 minutes; control reactions (no agent) were always carried out in parallel and the activity of the lysate containing the test substance compared directly to the control activity.

3. Erythroid cell proliferation

A CFU-E culture system was established and standardized for dog bone marrow aspirates. In essence, nucleated cells are isolated from the aspirated marrow by centrifugation in Ficoll-hypaque, placed in a defined culture medium, and incubated in microtiter wells for 48 hours at 39°C in a humidified incubator with a 4% carbon dioxide level. Following incubation, the plasma clots are placed on glass slides and stained. Erythroid colonies are then counted using the microscope; these colonies are defined as groups of eight or more hemoglobin staining cells.

To date the major activity in the laboratory has been to standardize this CFU-E assay. The first several months were devoted to obtaining appropriate culture

material in sufficient quantity to use for the experiments. Fifteen batches of bovine serum albumin were screened. One was found to have excellent activity in promoting CFU-E in dogs and has been purchased in quantity. Twenty-three different lots of fetal calf serum were screened. Two were found appropriate for the assay. Only one of these was commercially available in a reasonable amount and was obtained by our laboratory. These have been the two major variables in setting up the assay. The other necessary supplies, such as erythropoietin, bovine citrated plasma, etc., are obtained and have been working well.

As soon as the supply of appropriate culture materials was obtained, work began on standardizing the CFU-E assay. Variation in efficiency of culture techniques was a significant problem initially, and still presents problems occasionally. The major reasons for this are: (1) The cell separation technique of dog bone marrow in which nononuclear cells are separated from whole bone marrow by Ficoll-hypaque sedimentation has not yielded pure mononuclear cell populations. It has been both our experience and the experience of other investigators that this is uniformly difficult to accomplish due to size and shape similarities between mononuclear and non-mononuclear marrow precursor cells. We have, therefore, attempted to simply use the Ficoll-hypaque sedimentation to eliminate all mature red cells obtained from bone marrow aspiration and not to attempt at the present time to further purify bone marrow precursor cells. (2) The amount of nutrients available to the marrow precursor cells varies. We are attempting to diminish our cell inoculum for culture to better allow sufficient nutrients for cell maturation and development, and are proceeding with the addition of supplemental nutrients to our culture material to increase efficiency.

The relationship between number of cells in initial inoculum and number of colonies formed per 10^5 cells is shown in Figure 8. Erythropoietin dose response for the culture system is shown in Figure 9.

B. Comparative toxicity data on a limited number of compounds using all three test systems

1. Examination of vehicles for delivery of the benzene and toluene derivatives

Because methanol is routinely used for solubilizing the porphyrin in the heme synthetase reaction, and Tween-20 is used in preparing our homogenates for both the ALA synthetase and heme synthetase reactions, a systematic survey of vehicles was not undertaken for the heme synthesis enzymes.

The effects of six non-aromatic solvents, one detergent, and two mixtures of solvent and detergent were tested in the cell-free glcbin translation system. The solvents analyzed included: methanol, ethanol, ethylene glycol, diethyl ether, acetone and dioxane; the detergent was Tween-20 and the mixes of detergent and solvent were Tween-20 plus methanol or dioxane. The results of these assays are shown as dose-response curves in Figures 10 and 11 and in tabular form in order of decreasing potency in Tables VI and VII.

Not surprisingly, the solvents (and detergent) tested varied greatly in their effects on translation. In order of deceasing potency, the relative toxicities of the simple solvents at 2% concentration were: methanol, acetone, dioxane, ethanol, Tween-20, ethylene glycol, and ether. At 4% concentration of solvent, the relative toxicities were similar except that ether was more toxic than Tween-20. (Ethanol was not done at this concentration). Dose response studies on methanol, acetone, ethanol, ethylene glycol, and ether show increased inhibition at higher solvent concentrations. In three of the cases (methanol, ethanol, and ether), there was little inhibition until the solvent reached a specific concentration. The detergent, Tween-20, had essentially no effect on protein synthesis over the range of 0-2%, and only moderate inhibitory effect at 4%; the presence of Tween-20 neutralized somewhat the inhibitory effects of methanol and dioxane.

A comparable, but considerably less extensive survey was done of vehicles in the erythroid culture system. It became apparent that control cultures incubated with ethanol did better than our control cultures incubated without ethanol. This did not appear to be due to changes in pH but seemed to be related to increased nutrient availability.

Based on the above results, 1% ethyl alcohol was selected for use as a solvent to introduce the benzene and toluene derivatives into the translation and erythroid culture systems. In an effort to make the three systems as comparable as possible, the effect of 1% ethyl alcohol on ALA synthetase and heme synthetase will be tested, and ethanol will be used as the vehicle in this system as well, if feasible.

2. Purity of benzene and toluene derivatives used in toxicity studies

The aromatic compounds studied came primarily from commercial sources, principally Aldrich Chemical Company. Some compounds unavailable from commercial sources were obtained from the Division of Experimental Therapeutics at WRAIR. We tested for impurities by subjecting each compound to thin-layer chromatography using two different solvent systems: benzene-ethyl acetate 1:4, and acetonitrile. Each compound tested showed only a single spot on migration on pre-coated silica gel plates (E.M. Laboratories, 500 Executive Blvd., Elmsford, NY 10523).

3. Effect of benzene on heme synthesis and globin synthesis

In the concentration range 10^{-6} to 10^{-3}, benzene had little effect on either ALA synthetase or heme synthetase (Table XI).

Figure 12 and Table VII show the dose-response results obtained thus far for benzene in the globin translation system. Because of the low solubility of benzene in water (8.9×10^{-3} M) assays were performed for benzene alone over the concentration range 10^{-6}-10^{-3} M and benzene in 4% diethyl ether over the range 10^{-6}-10^{-1} M. (Ether at the 4% level inhibits activity by about 20% -Figure 10.) The results show that benzene has no deleterious effect on protein synthesis in the cell-free system used here and indeed, appears to stimulate amino acid incorporation at all concentrations tested.

4. Effect of the dinitrobenzenes on heme synthesis and globin synthesis

Figure 13 shows a comparison of the activities of

the dinitrobenzenes on ALAS and HS activities. One may note that in concentration ranges greater than 10^{-5} mol/l, as HS activity increases ALAS activity decreases.

Figure 14 and Table VIII show the results obtained when the three isomers of dinitrobenzene were tested on the rabbit cell-free globin synthesis system. All three compounds are soluble in water at the concentrations tested. Inspection of the results show that 1,3-DNB has essentially no effect over the range tested (6×10^{-8} - 6×10^{-4} M), whereas 1,4-DNB and 1,2-DNB inhibit at most concentrations.

5. Effect of the dinitrotoluenes on heme synthesis, globin synthesis, and erythroid cell proliferation

Figure 15 gives the comparison of the dinitrotoluenes' effect on ALA synthetase and heme synthetase. At concentrations similar to the dinitrobenzenes, the effect is not as great, which suggests that there may be some interaction between the methyl and nitro groups that offset the enhancing-inhibiting effects. A similar trend may be noted with trinitrotoluenes and amino-dinitrotoluenes as shown in Figure 16. The addition of an amino group to the aromatic ring appears to lessen the decrease in ALAS activity.

When five of the six possible dinitrotoluene (DNT) homologues were tested (only 3,5-DNT has yet to be tested) in the cell-free globin translation system, the dose-response data shown in Figure 17A and 17B and Table IX were obtained.

Testing was performed over the concentration range of 10^{-12} to 10^{-4}. All of the DNT's tested inhibited translation to some extent although to varying degrees and at different concentrations. Variation of inhibition with concentration of DNT did not seem to be a simple relationship. For many of the compounds, inhibition appeared to be more pronounced at their highest and/or lowest concentrations. For example, inhibition of protein synthesis by 2,3-DNT and 2,5-DNT were more pronounced at lower concentrations than at higher ones. For 2,3-DNT, relative activity of globin translation was 80% for 10^{-12} M vs 109% for 10^{-8} M, 94% for 10^{-6} M, and 91% for 10^{-4} M. For 2,5-DNT, relative activity of

globin translation was 70% for 10^{-12}M and 68% for 10^{-8}M, vs 86% for 10^{-6}M and 92% for 10^{-4}M. For other compounds, inhibition of protein synthesis was more pronounced at higher concentrations than at lower ones. For example, relative activity of globin translation was 70% for 10^{-4}M 2,6-DNT and 67% for 10^{-6}M, vs 83% and 94% for 10^{-8} and 10^{-12}M concentrations of this compound. From the data in Table IX, it appears that the order of inhibitory effectiveness of the dinitrotoluenes tested is: (1) most inhibitory: 2,6-DNT (high doses) and 2,5-DNT (low doses); (2) intermediately inhibitory: 3,4-DNT and 2,3-DNT; and (3) least inhibitory: 2,4-DNT.

Preliminary observations of the effects of graded concentrations of 2,6-DNT and of 3,4-DNT on erythroid colony formation are shown in Figures 18 and 19, respectively. For 2,6-DNT, inhibition of colony formation was present at each concentration tested (figure 18). Inhibition increased with increasing dose of the 2,6-DNT (21% inhibition at 10^{-6}M; 34% inhibition at 10^{-5}M; 38% inhibition at 10^{-4}M). For 3,4-DNT, inhibition of colony formation was not present at 10^{-7}M or 10^{-6}M; however, there was 33% inhibition at 10^{-5}M.

6. Correlation of data obtained using two or three of the test systems for specific compounds

Little correlation can be done at this time among systems for two reasons: (a) The erythroid culture system has only recently been standardized and characterized, and only two compounds have been examined with this system so far. (b) Results of the studies of effects of the various compounds on protein synthesis are not entirely clear. The fact that both high and low levels of the benzene and toluene derivatives inhibit translation, whereas intermediate levels do not, must be shown to be a real phenomenon and not an artifact produced by a non-optimized system.

C. Utilization of dose-toxicity studies of a large number of nitrotoluenes and their analogues on ALA synthetase and heme synthetase to determine which groups of compounds may show significant structure-activity relationships

Table X shows preliminary screening studies of benzene and toluene derivatives. Benzene, toluene,

nitrobenzene, nitrotoluene and the chlorotoluenes (o-, m-, and p-) demonstrate only minimal effects on ALA synthetase and heme synthetase. 2-amino-4-nitrotoluene and 1-chloro-2-nitrobenzene show slightly more pronounced alterations in enzyme activity. The dinitrotoluenes, trinitrotoluenes, and amino-dinitrotoluenes produce, for the most part, pronounced decreases in ALA synthetase activities and increases in HS activities, with the degree of enzyme alteration varying with the particular structure. Hexachlorobenzene appears to increase heme synthetase activity without significantly decreasing ALA synthetase activity; in fact, it increases ALA synthetase activity at a concentration of 10^{-3} M. Aniline's effect on ALA synthetase is minimal; it produces a moderate increase in heme synthetase at high doses.

D. Summary of work completed so far

1. Experimental results

Three test systems - heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system, and erythroid cell proliferation in a plasma clot culture system have been established and standardized. For the enzyme systems, studies have been done to show the stability, at -70°C, of the enzymes.

Vehicles used for delivery of the benzene and toluene derivatives were examined for their effects on the globin translation and erythroid culture systems. Based on the results obtained in this study, 1% ethyl alcohol was selected for use as a solvent to introduce the benzene and toluene derivatives into these two systems. Because methanol is routinely used for solubilizing the porphyrin in the heme synthetase reaction, and Tween-20 is used in preparing our homogenates for both the ALA synthetase and heme synthetase reactions, a systematic survey of vehicles was not undertaken for the heme synthesis enzymes.

The effects of benzene, the dinitrobenzenes, the dinitrotoluenes, and the tri-substituted toluenes on heme synthesis and globin synthesis were studied. Benzene was shown to have little deleterious effect on

either the enzymes of heme synthesis or globin translation. Ortho- and para- dinitrobenzene were shown to affect heme and globin synthesis more than meta- dinitrobenzene. Examination of the effects of the dinitrotoluenes on heme synthesis and globin translation revealed that (a) alterations of enzyme activity do not seem to be related solely to the relative positions of the two nitro groups with respect to each other, and (b) in some cases, one enzyme system was sensitive to a particular compound while the other was not.

Dose-toxicity studies of the effect of 23 benzene and toluene derivatives on ALA synthetase and heme synthetase were examined. The most pronounced effects were seen with the dinitrobenzenes, the dinitrotoluenes, the trinitrotoluenes, the amino-dinitrotoluenes, and hexachlorobenzene. Less pronounced effects were seen with benzene, toluene, nitrobenzene, nitrotoluene, the chlorotoluenes, 2-amino-4-nitrotoluene, and 1-chloro-2-nitrobenzene, Table XI.

2. Problem areas

The major problem area at the present time is standardization of the three test systems so that results for each system are not only qualitatively consistent, but also quantitatively reproducible. In standardizing the system, particular attention will have to be paid to the globin synthesis studies, in order to determine whether inhibition by the lowest concentration (10^{-12}M) of toluene derivatives and the frequent occurrence of inhibition at both high and low (but not intermediate) concentrations of the derivatives is real or artifactual.

A second problem area is that the toxicity studies have only been done once for each compound. Several repeat studies will have to be performed for each compound.

3. Usefulness of this approach to in vitro toxicity testing

It is too early to determine whether the approach we are using will be of practical use for in vitro toxicity testing. Hopefully, we will be able to show that the three systems give similar data on relative toxicity of a given compound. If that is the case, then

the simplest of the assays will have predictive value, and can be used as a preliminary screen.

Project 3M161102BS01 RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 145 The Effects of Specific Environmental Pollutants on the Biosynthetic Functions of Mammalian Cells In Vitro; A Search for Structure/Activity Relationships

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Figure 1.

Linearity of ALA synthetase reaction with respect to time

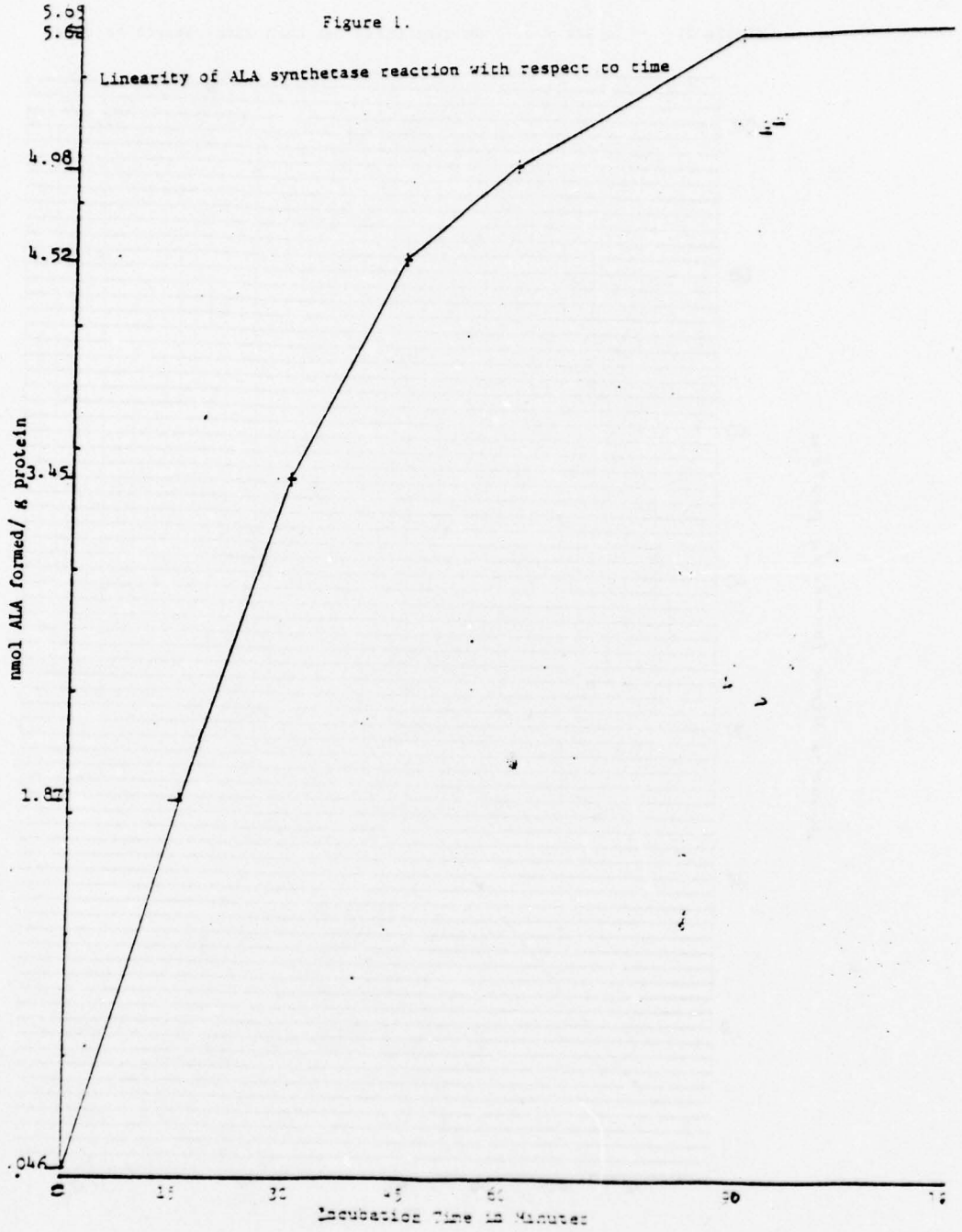


Figure 2. Linearity of heme synthetase reaction with respect to time

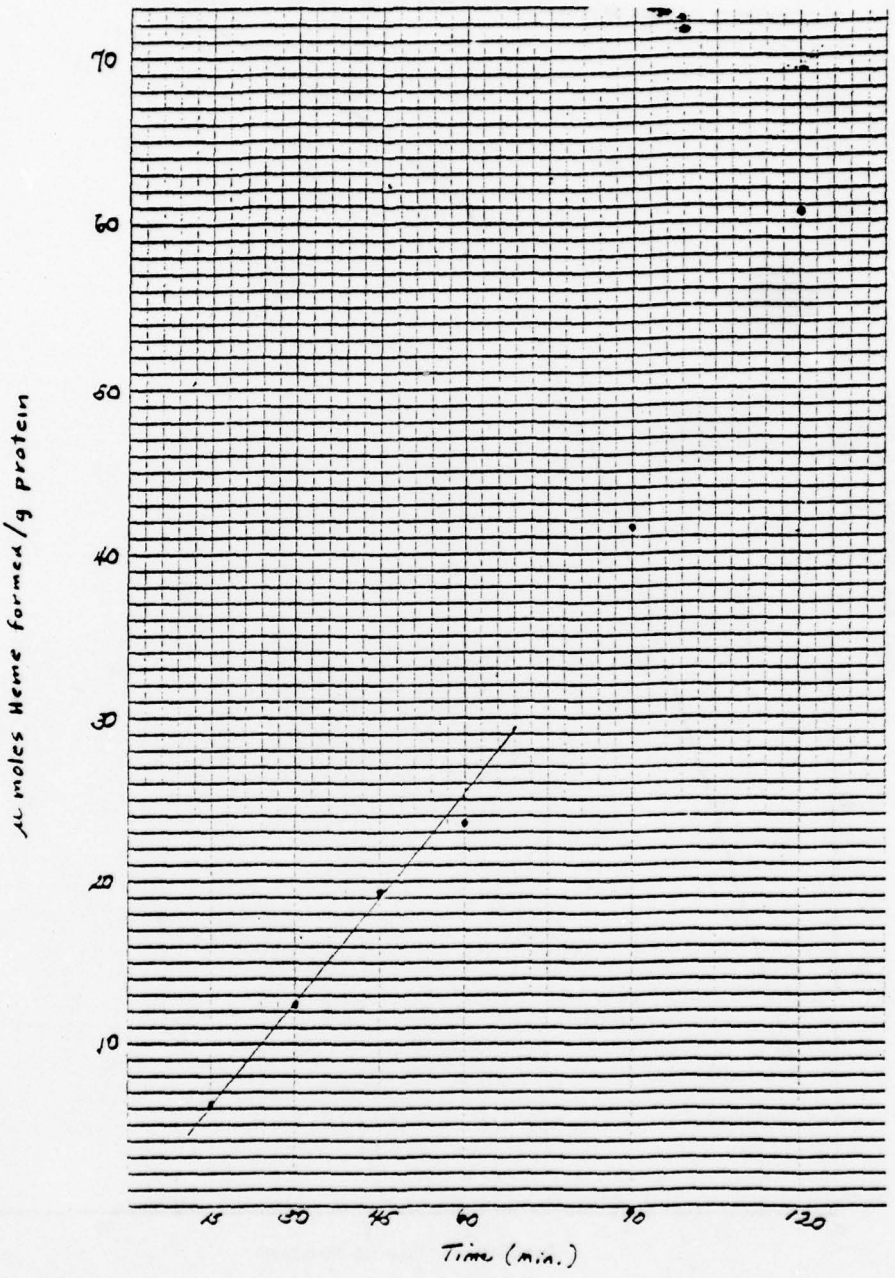


Figure 3. Relation of rate of ALA synthetase reaction to protein concentration of rat liver homogenate and to concentration of alphaketoglutarate.

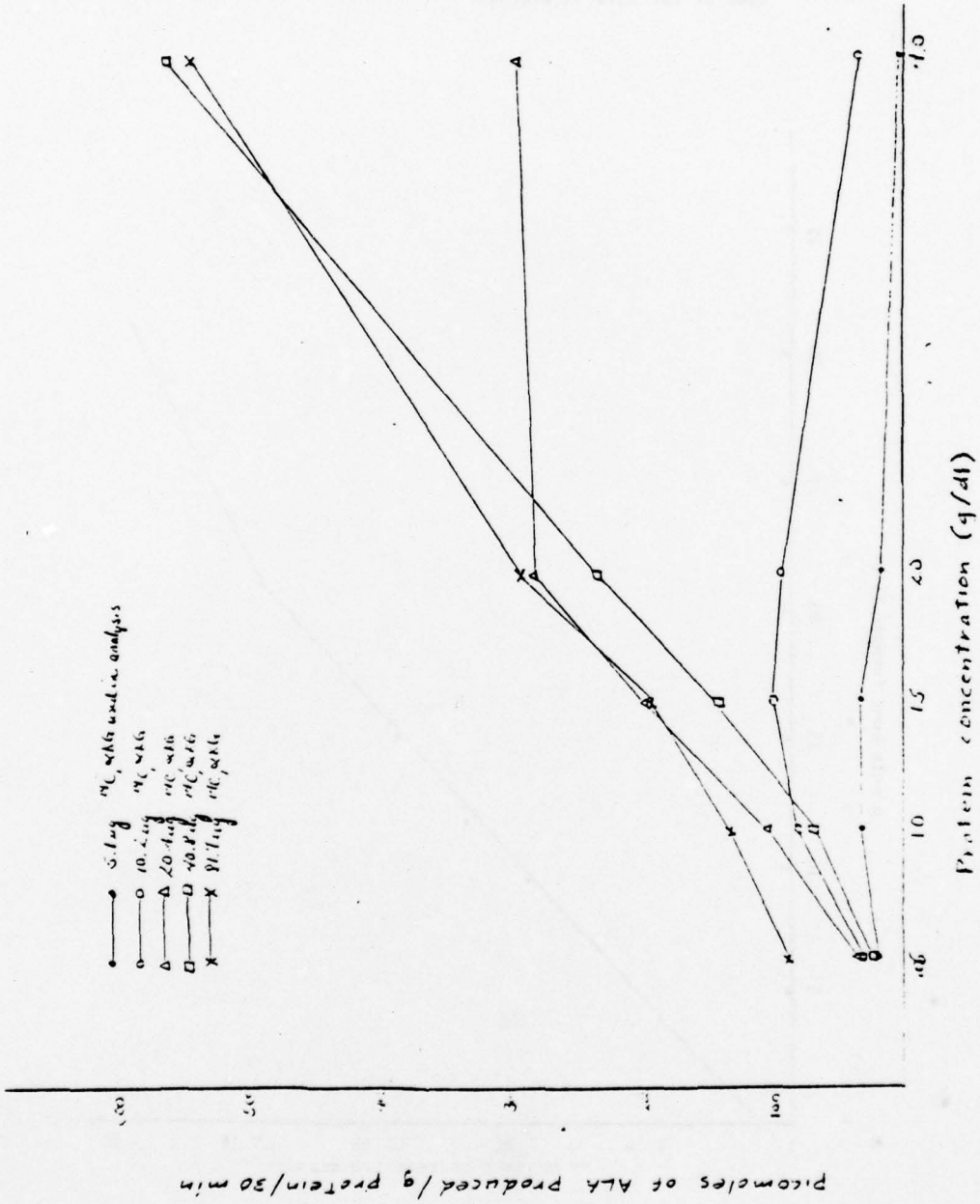


Figure 4. Relationship between heme synthetase activity and protein concentration of rat liver homogenate

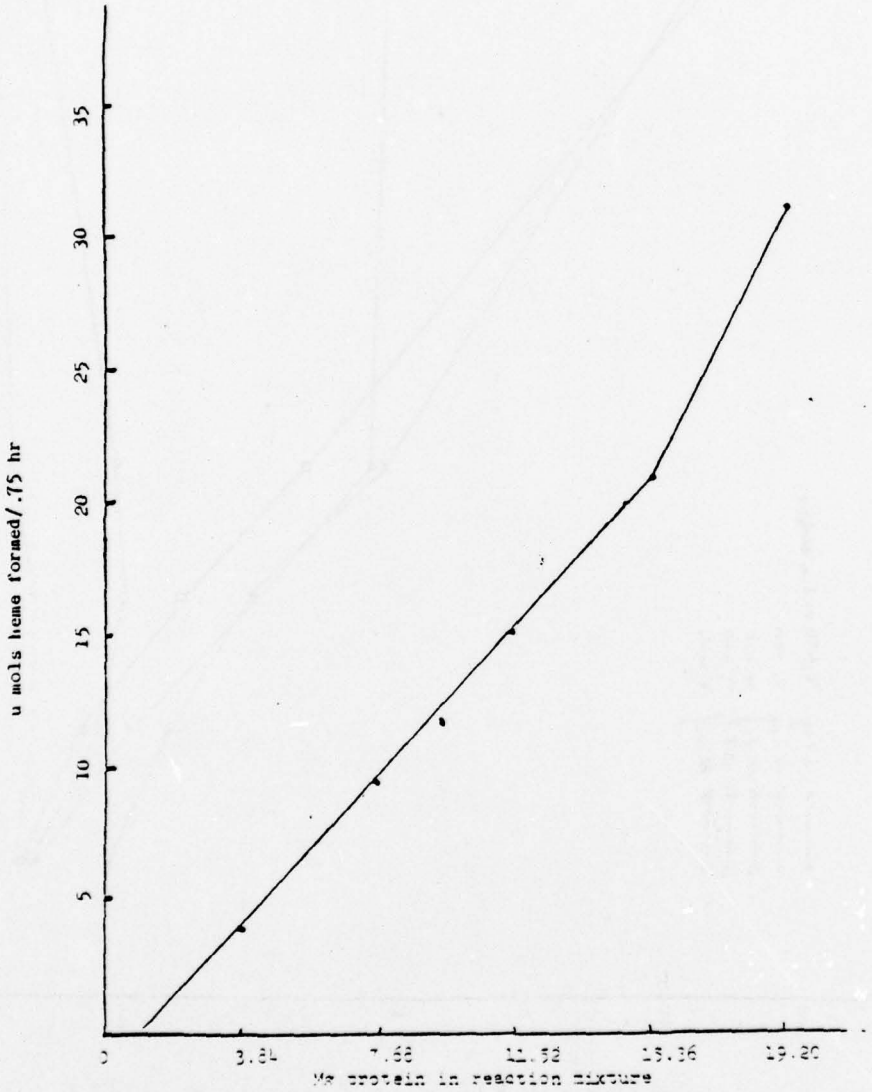


Table I. Day-to-day and within-day variation of ALA synthetase activity on pooled rat liver homogenate-sonicates. Aliquots of pooled homogenate were frozen at -70°C and examined at days 2, 3, 7, and 20.

	Day 0	Day 2	Day 3	Day 7	Day 20
	3.70	3.79	3.80	3.77	3.49
	3.65	3.81	3.74	3.86	3.30
	3.63	3.63	3.55	3.84	3.48
	3.63	3.61	3.71	3.88	3.46
	3.63	3.64	3.70	3.69	3.45
	3.63	3.61	3.63	3.83	3.31
	3.63	3.67	3.89	3.72	3.25
	3.64	3.78	3.72	3.82	3.33
	3.61	3.68	3.53	3.88	3.32
	3.60	3.69	3.69	3.99	3.28
Mean	3.64	3.69	3.70	3.83	3.37
S.E.M.	.009	.024	.034	.027	.029

nmol/g protein/0.5 hr

Table II. Within-day reproducibility of heme synthetase. Homogenate-sonicate from seven individual livers were examined.

	Homogenate						
	1	2	3	4	5	6	7
umol Heme formed per 45 min. per g protein	17.8	13.6	11.9	21.3	20.1	22.9	13.3
	16.2	11.4	12.9	27.9	22.0	19.2	13.2
	17.2	11.9	12.6	26.1	21.7	23.0	12.5
		12.1	12.2	25.3	21.8	23.3	
		12.5	13.8	27.3	20.0		
		12.0	11.7	24.1			
			12.4				
			11.7				
Mean	17.1	12.3	12.4	25.3	21.1	22.1	13.0
S.E.M.	.47	.31	.25	.98	.44	.97	.25

Table III.

STABILITY OF HEME SYNTHETASE

	umol/g protein/45 min.			
RAT 1:	Day 1	Day 24		Day 58
	15.5	14.9		10.9
	17.3	18.2		12.4
Mean:	16.4	16.7		11.7
RAT 2:	Day 1	Day 25	Day 27	Day 28
	11.7	11.9	11.6	11.1
	10.3	11.6	12.0	10.6
		11.2		
Mean:	11 0	11.6	11.8	10.9

Figure 5. Variation of heme synthetase activity with iron concentration

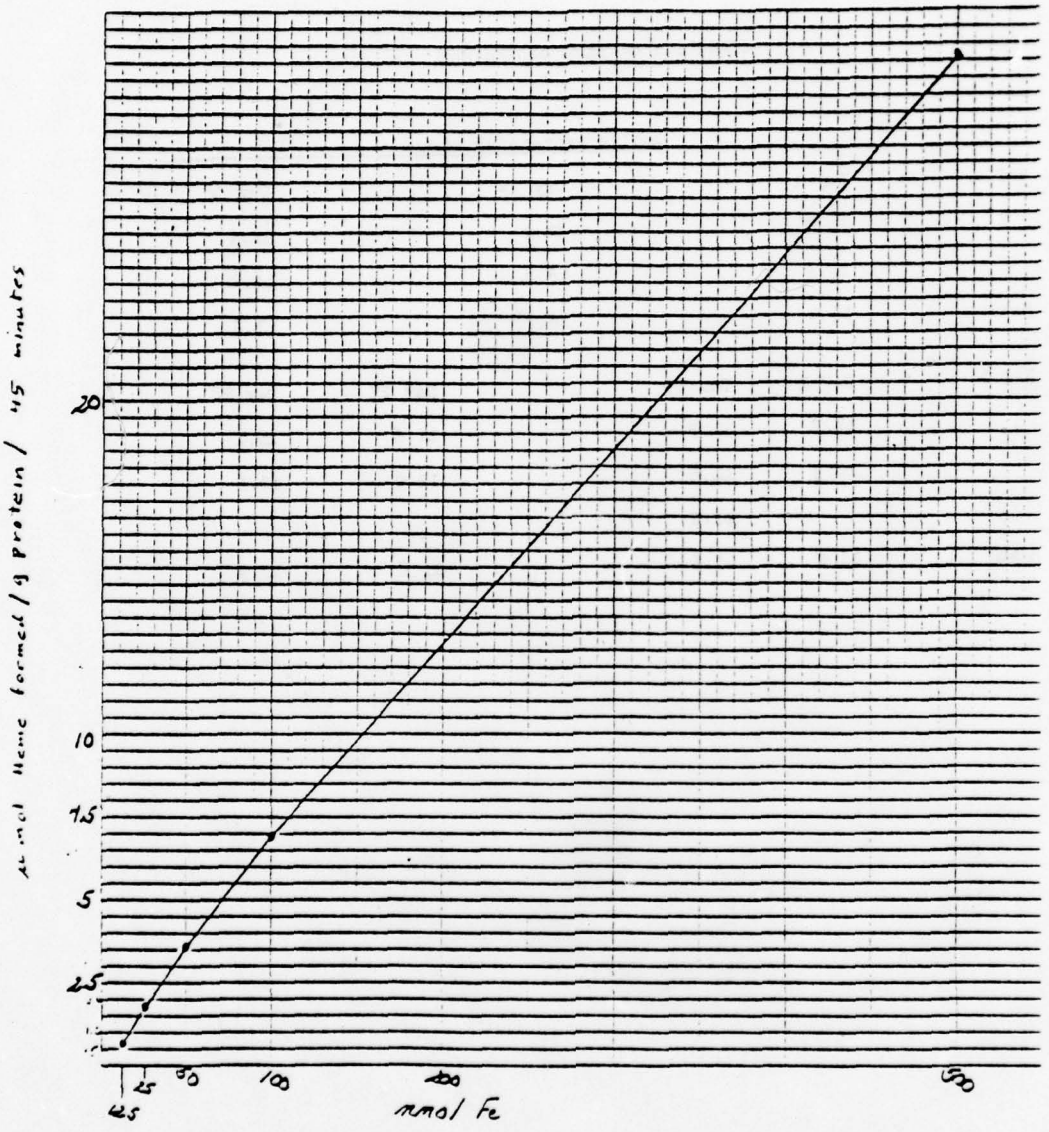


Figure 6. Variation of heme synthetase activity with porphyrin concentration

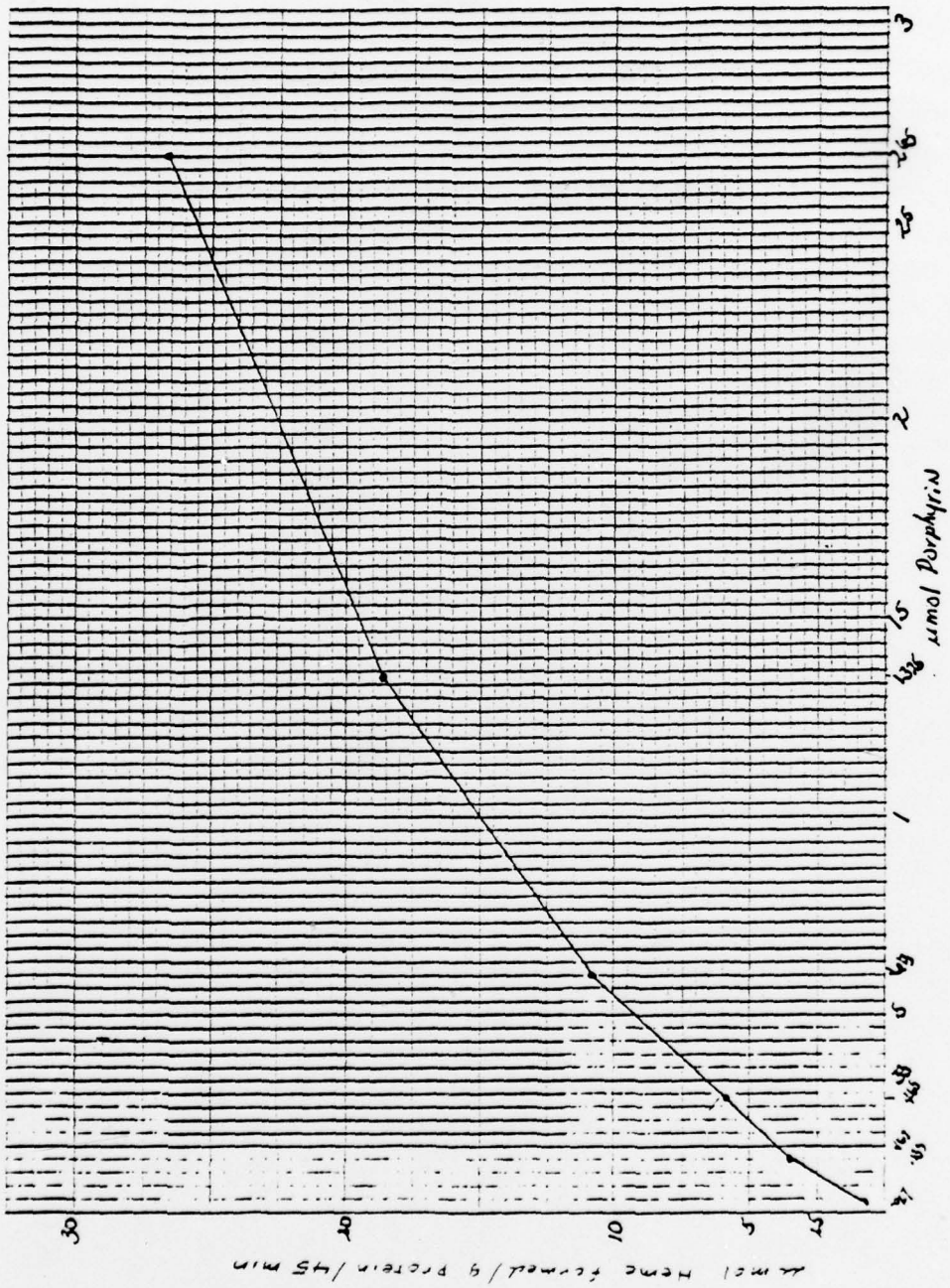


Table IV.

Effect of Added Heme on Heme Synthetase Activity

nmol of Added Heme	umol of Heme formed/g/45 min.
0	40.1
4	39.7
8	38.4
20	33.4
40	27.4
80	36.9
160	31.1
200	28.5

Figure 7

Rate of Cell-Free Incorporation of Radiolabeled Leucine into Protein by Rabbit Reticulocyte Lysate System

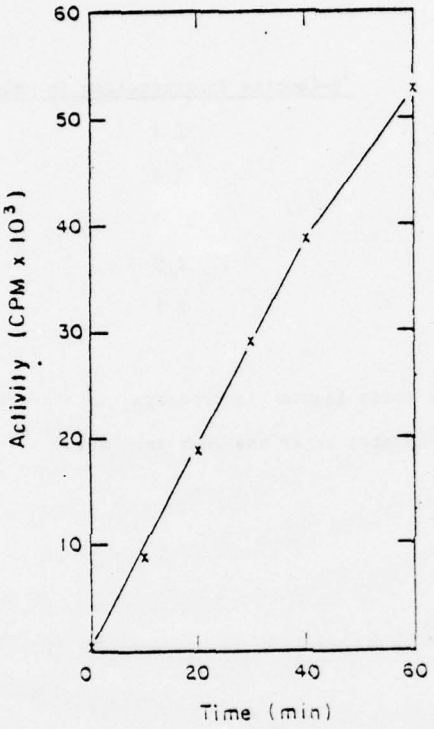


Table V

Cell-Free Synthesis of Protein by Five Different Preparations of Rabbit Reticulocytes

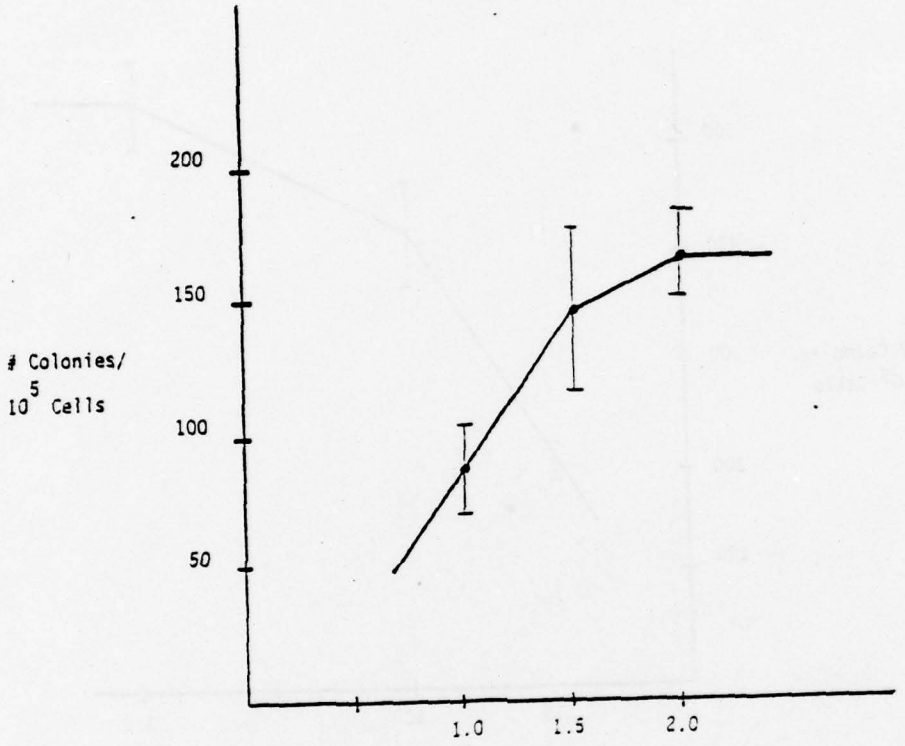
Lysate	^{3}H-Leucine Incorporation (p moles x 10^{2})
1	2.3
2	2.8
3	15.0
4	2.8
5	2.7

Activity is expressed as pmols leucine incorporated into protein per 50 µl reaction mixture (20 µl lysate) after one hour incubation.

Figure 8

Effect of Initial Inoculum on

Colony Numbers

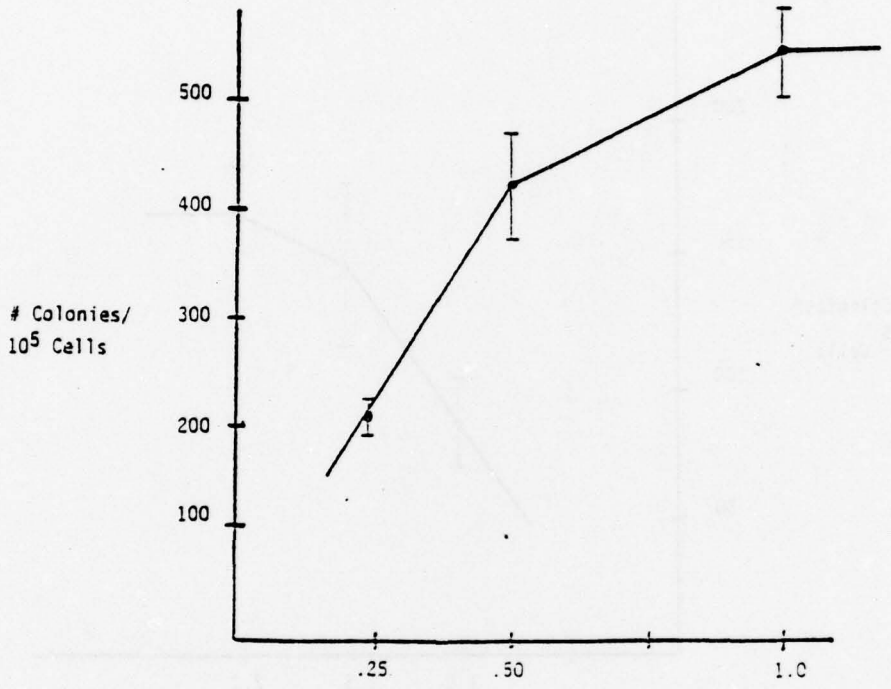


No. of Cells in Initial Inoculum	No of Colonies/10^5 Cells
1.0×10^5/ml	84 (± 14)[a]
1.5×10^5	146 (± 27)
2.0×10^5	164 (± 16)

[a] Value = mean (± S.E.M.) of 3 replicate cultures.

Figure 9

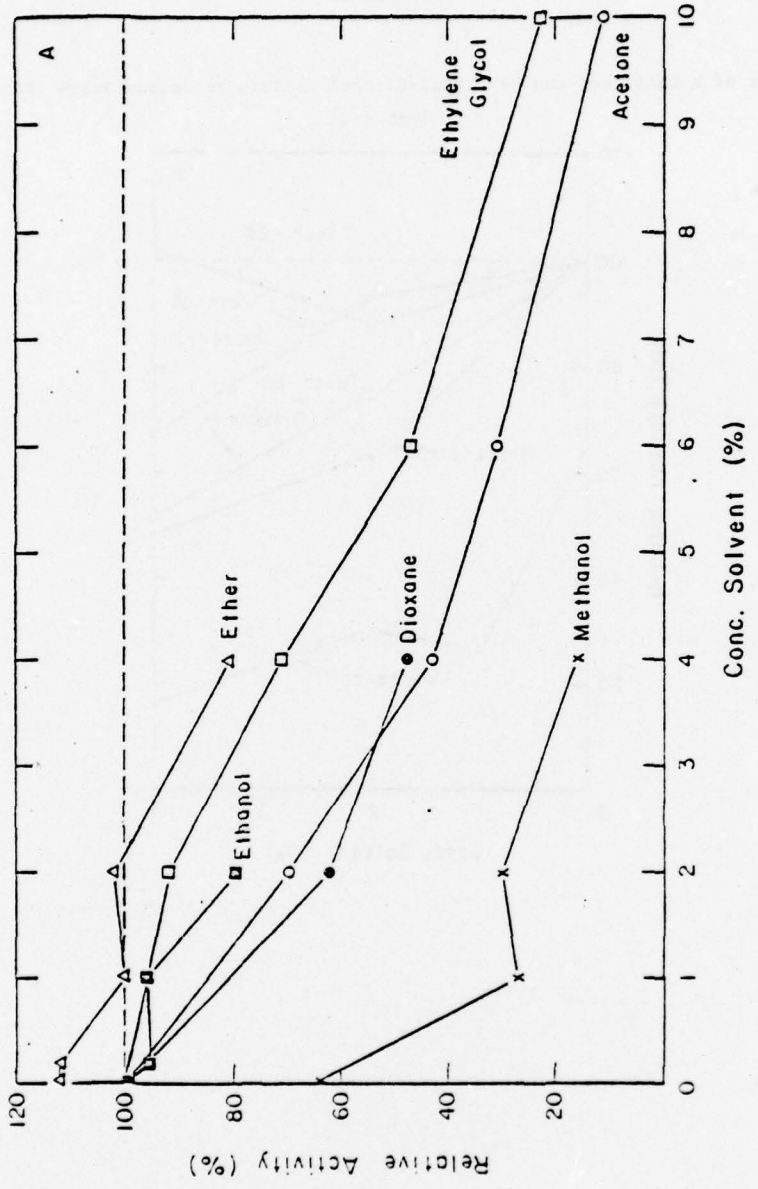
Erythropoietin Dose Response



(Erythropoietin)	No. of colonies/10^5 cells (initial inoculum)
0.25 units/ml	205 (± 20)[a]
0.50	425 (± 52)
1.00	550 (± 42)

[a] Value = mean (± S.E.M.) of 3 replicate cultures.

Figure 10



Effect of Various Organic Solvents on Cell-Free Synthesis of Rabbit Globin

Figure 11

Effect of a Detergent and Detergent-Alcohol Mixture on Reticulocyte Protein Synthesis

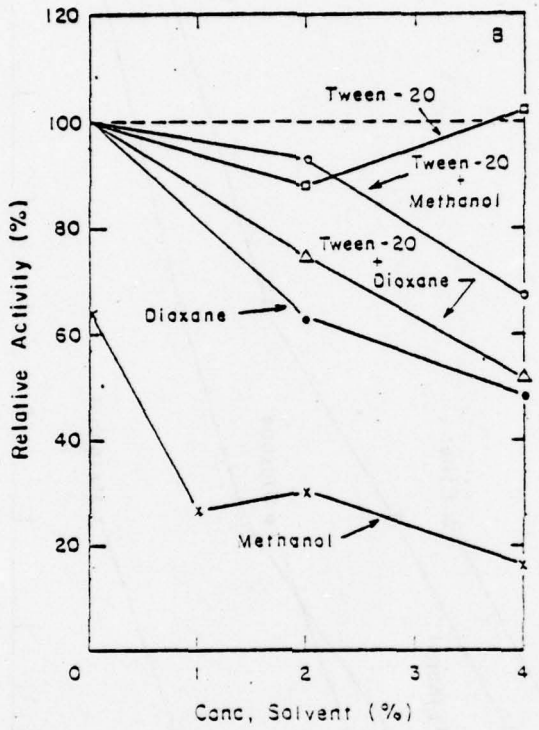


Table VI

Effect of Various Solvents and a Detergent on Cell-Free Synthesis of Rabbit Globin: Relative Activity of Incorporating System as a Function of Solvent/Detergent Concentrations

	Relative Activity (%)					
Solvent	.2%	1%	2%	4%	6%	10%
methanol	-	27	30	16	-	-
acetone	-	-	54	43	31	11
dioxane	-	-	63	48	-	-
dioxane + 2% Tween-20	-	-	75	52	-	-
ethanol	95	96	80	-	-	-
methanol + 2% Tween-20	-	-	93	63	-	-
ethylene glycol	-	-	92	71	48	23
Tween-20	-	-	88	102	-	-
ether	112	100	102	81	-	-

Figure 12

Effect of Benzene on Cell-Free Protein Synthesis by Rabbit Reticulocyte Lysate

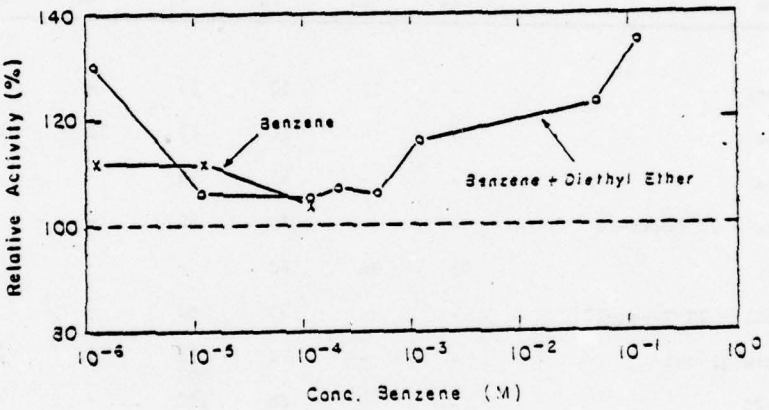


Table VII

Effect of Benzene on Cell-Free Protein Synthesis by Rabbit Reticulocyte Lysate

Conc. of Benzene (M)	Relative Activity (%) in Diethyl Ether	in H_2O
1.78×10^{01}	135	-
7.12×10^{-2}	123	-
1.78×10^{-2}	116	-
7.12×10^{-3}	106	-
3.56×10^{-3}	107	-
1.78×10^{-3}	105	104
1.78×10^{-4}	106	112
1.78×10^{-6}	130	112

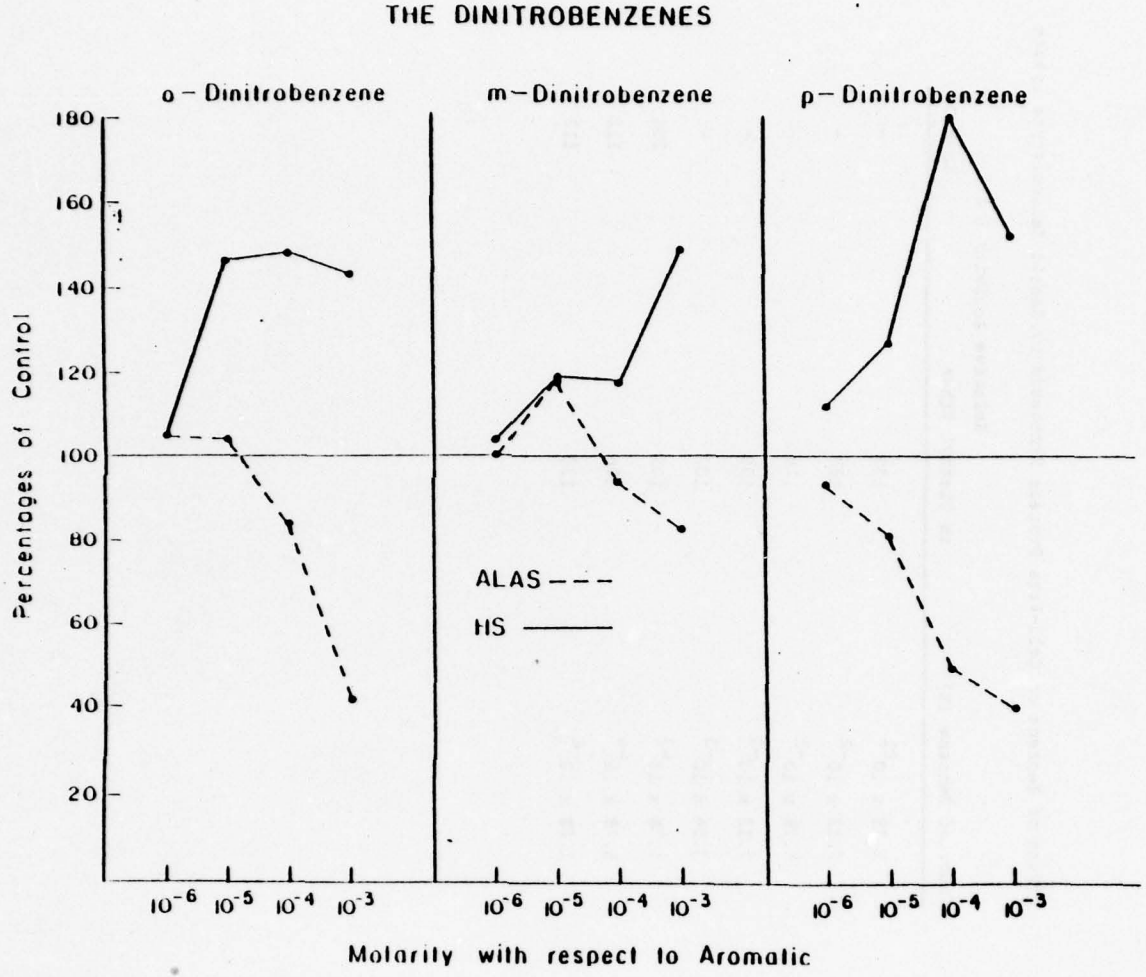

THE DINITROBENZENES
o – Dinitrobenzene
m – Dinitrobenzene
p – Dinitrobenzene
Percentages of Control
180
160
140
120
100
80
60
40
20
ALAS - - -
HS ——
10^{-6} 10^{-5} 10^{-4} 10^{-3}
Molarity with respect to Aromatic

Figure 13

Figure 14

Effect of Three Isomers of Dinitrobenzene (DNB) on Cell-Free Synthesis of Rabbit Globin

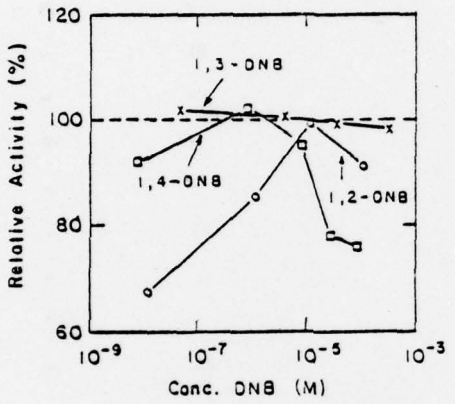
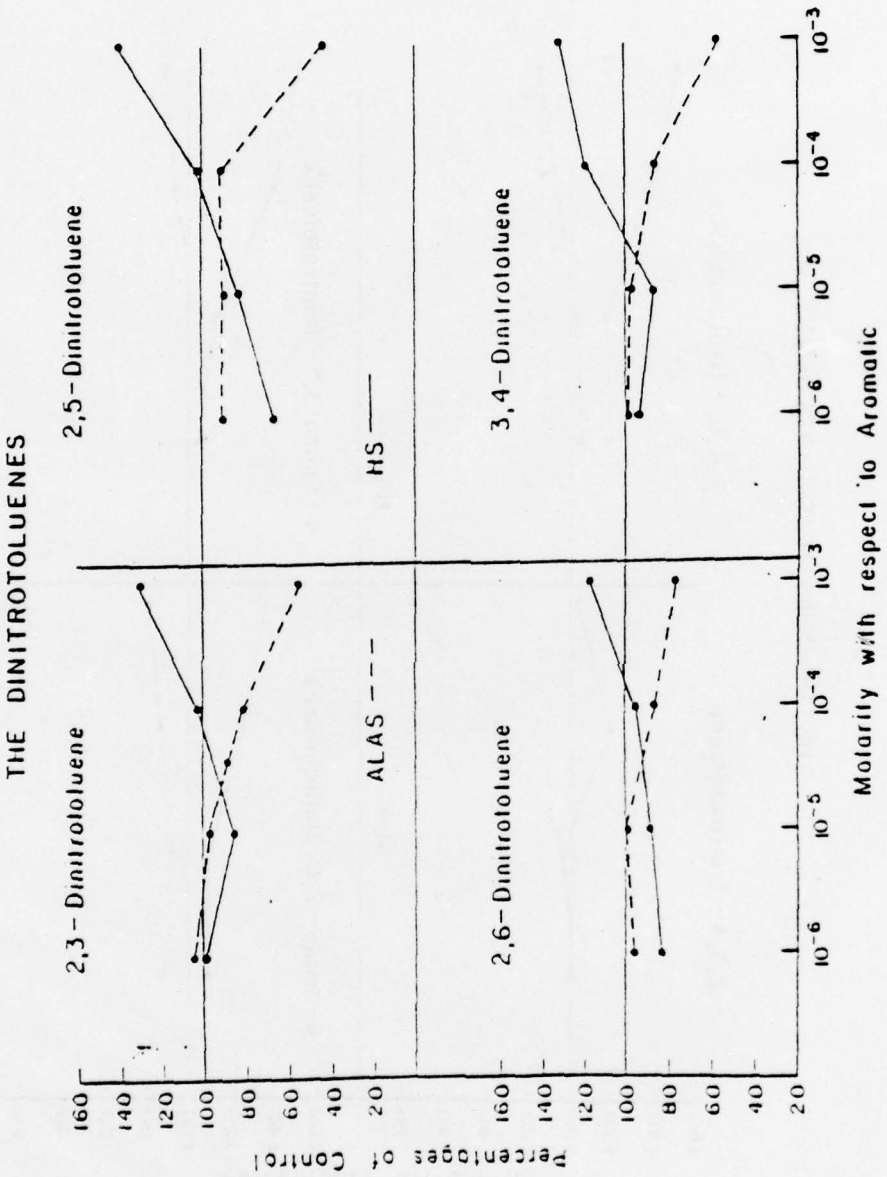


Table VIII

Effect of Three Isomers of Dinitrobenzene (DNB) on Cell-Free Synthesis of Rabbit Globin

Conc. Dinitrobenzene (M)	Relative Activity (%) 1,2-DNB	1,3-DNB	1,4-DNB
5.9×10^{-4}	-	98	-
1.8×10^{-4}	91	-	-
9.4×10^{-5}	-	-	76
5.9×10^{-5}	-	99	-
4.7×10^{-5}	-	-	78
1.8×10^{-5}	99	-	-
9.4×10^{-6}	-	-	95
5.9×10^{-6}	-	100	-
1.8×10^{-6}	85	-	-
9.4×10^{-7}	-	-	102
5.9×10^{-8}	-	102	-
1.8×10^{-8}	67	-	-
9.4×10^{-9}	-	-	92

Figure 15


THE DINITROTOLUENES
2,3-Dinitrotoluene
2,5-Dinitrotoluene
2,6-Dinitrotoluene
3,4-Dinitrotoluene
HS ——
ALAS - - -
Percentages of Control
Molarity with respect to Aromatic

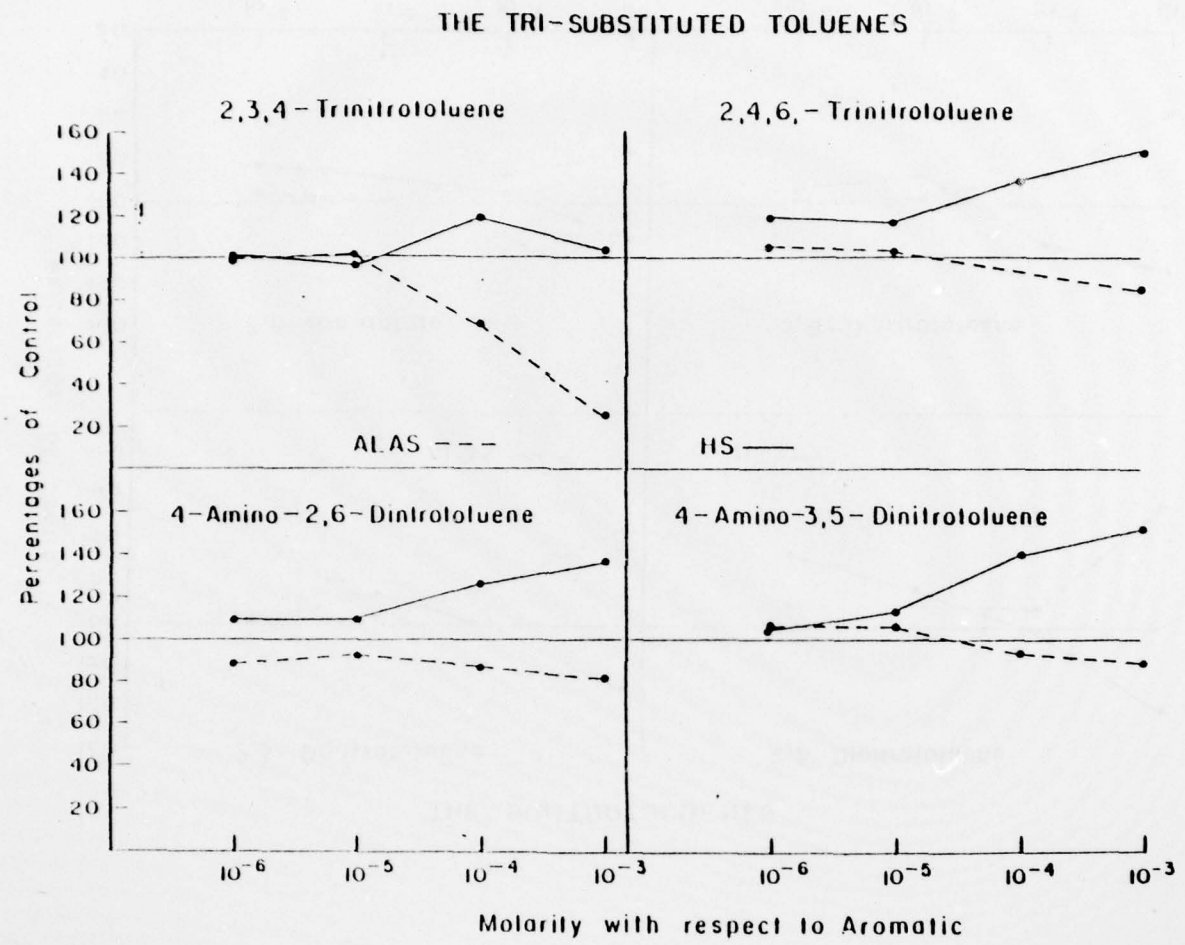

THE TRI-SUBSTITUTED TOLUENES
2,3,4 - Trinitrotoluene
2,4,6, - Trinitrotoluene
4 - Amino - 2,6 - Dinitrotoluene
4 - Amino-3,5 - Dinitrotoluene
ALAS - - -
HS ——
Percentages of Control
160
140
120
100
80
60
40
20
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Molarity with respect to Aromatic

Figure 16

Figure 17

Effect of Various Dinitrotoluene Isomers on In vitro Protein Synthesis by Rabbit Reticulocyte Lysates

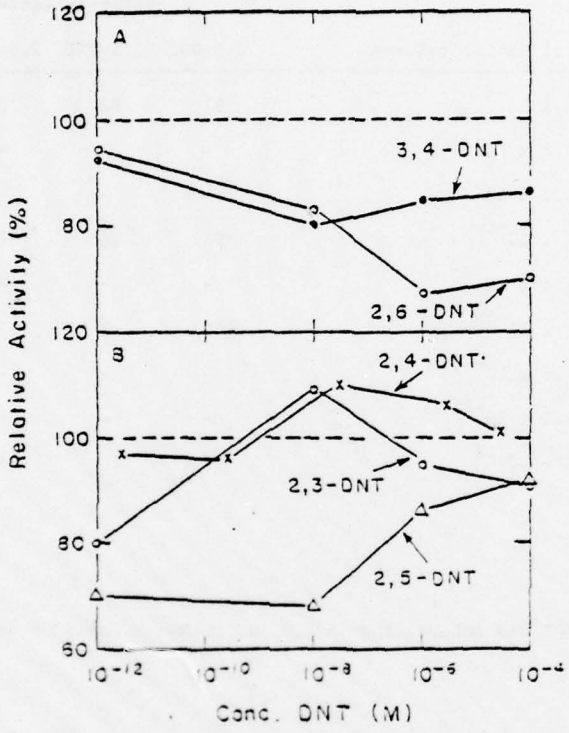


Table IX

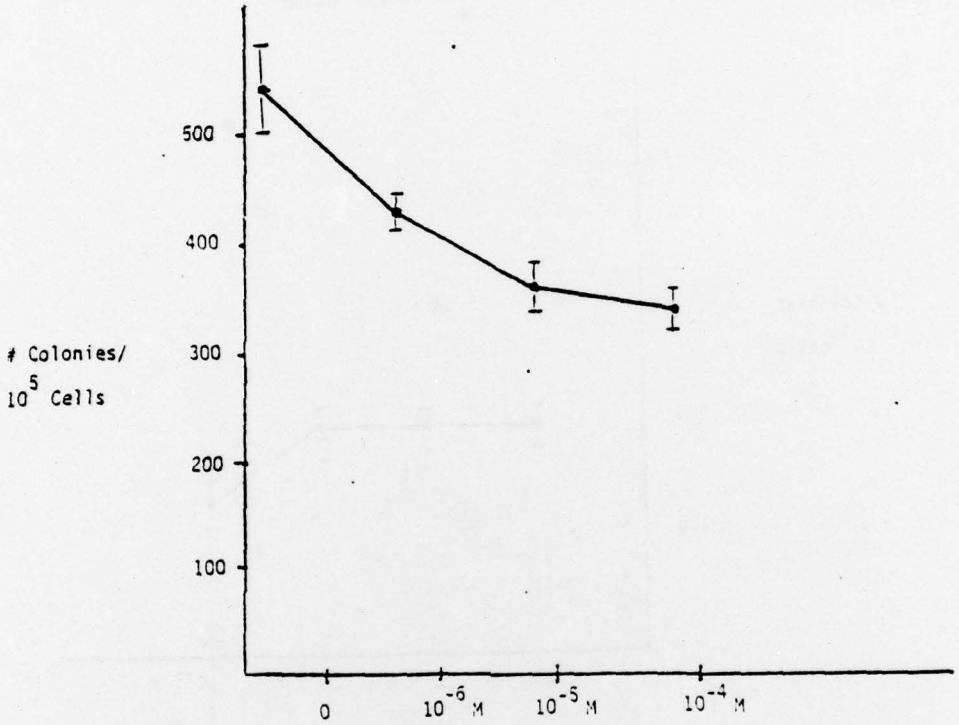
Effect of Various Dinitrotoluene Isomers on *In vitro* Protein Synthesis by Rabbit Reticulocyte Lysates

Conc. of Dinitrotoluene	Relative Activity (%)				
	2,3-DNT	2,5-DNT	2,6-DNT	3,4-DNT	2,4-DNT[+]
1×10^{-4}	91	92	70	86	-
4.6×10^{-5}	-	-	-	-	101
4.6×10^{-6}	-	-	-	-	106
1×10^{-6}	94	86	67	84	-
4.6×10^{-8}	-	-	-	-	110
1×10^{-8}	109	68	83	80	-
4.6×10^{-10}	-	-	-	-	96
4.6×10^{-12}	-	-	-	-	-
1×10^{-12}	80	70	94	92	97

[+]2,4-DNT was solubilized in H_2O, all other isomers in ethanol.

Figure 18

Effect of Concentration of 2,6 DNT on Colony Number



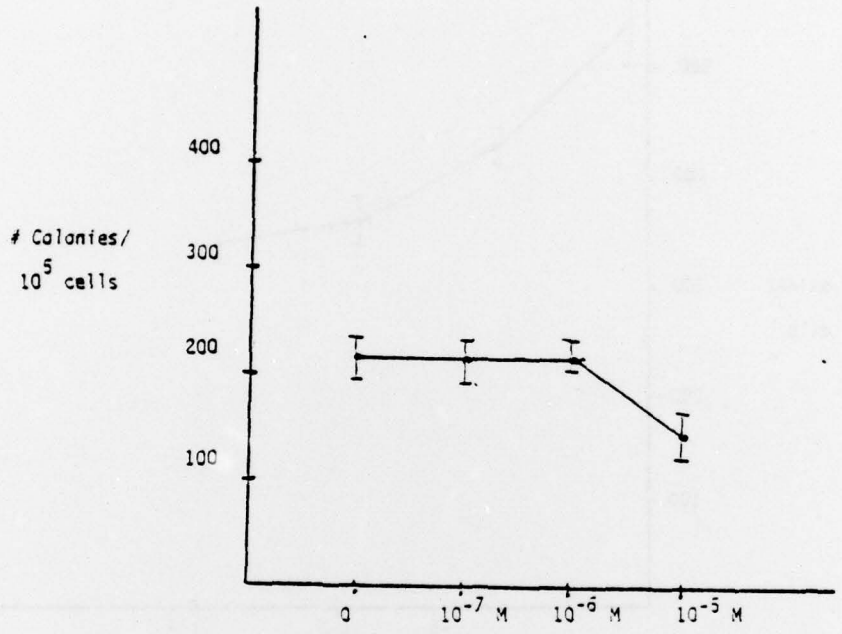
(2,6 DNT)	No. of Colonies/10^5 cells (initial inoculum)	
0	550 (± 42)[a]	
10^{-6} M	433 (± 13)	21% inhibition
10^{-5} M	365 (± 15)	34% inhibition
10^{-4} M	339 (± 19)	38% inhibition

[a] Value = mean (± S.E.M.) of 3 replicate cultures

Figure 19

Effect of Concentration of 3,4 DNT

on Colony Number



final (3,4 DNT)	#/10^5	
-	217 (± 19)[a]	
10^{-7} M	215 (± 19)	
10^{-6} M	217 (± 9)	
10^{-5} M	145 (± 23)	33% innibition

[a] Value = mean (± S.E.M.) of three replicate cultures

Table X

COMPARISON OF TOXICITY STUDIES

Compound	96-hr. LC50 (mg/liter) in Minnows	ALAS (Percentage of Control)	HS
Toluene	12.6	96	92
4-Nitrotoluene	49.9	84	93
2,3-Dinitrotoluene	1.9	55	130
2,4-Dinitrotoluene	32.5	92	87
2,5-Dinitrotoluene	1.3	43	139
2,6-Dinitrotoluene	19.8	77	117
3,4-Dinitrotoluene	1.5	56	131
2,4,6-Trinitrotoluene	2.4	86	150
2-Amino-4-Nitrotoluene	71.3	89	97
4-Amino-2,6-Dinitrotoluene	6.9	82	137
4-Amino-3,5-Dinitrotoluene	13.1	89	152

NOTE: The lower the LC50 number obtained by Ft. Detrick, the more drastic the change in the percentage of control we obtained for ALAS (usually a decrease) and for HS (usually an increase).

Table XI

Concentration of Aromatic	Percentages of Control							
	10^{-3} mol/1		10^{-4} mol/1		10^{-5} mol/1		10^{-6} mol/1	
Test	ALAS	HS	ALAS	HS	ALAS	HS	ALAS	HS
Aromatic								
Benzene	95	86	104	91	107	94	107	89
Toluene	96	92	104	86	93	91	100	89
Nitrobenzene	86	111	108	104	110	100	81	105
o-Dinitrobenzene	43	143	84	148	104	146	105	105
m-Dinitrobenzene	83	149	94	118	118	119	100	104
p-Dinitrobenzene	41	152	50	180	81	127	93	112
4-Nitrotoluene	84	93	95	99	96	107	96	113
2,3-Dinitrotoluene	55	130	81	103	97	85	104	99
2,4-Dinitrotoluene	92	87	100	80	102	92	99	96
2,5-Dinitrotoluene	43	139	91	102	89	82	90	66
2,6-Dinitrotoluene	77	117	87	96	100	89	97	84
3,4-Dinitrotoluene	56	131	86	119	97	87	99	94
2,3,4-Trinitrotoluene	26	104	69	119	101	96	99	100
2,4,6-Trinitrotoluene	86	150	93	136	103	117	105	119
4-Amino-2,6-Dinitrotoluene	82	137	87	126	93	110	89	109
4-Amino-3,5-Dinitrotoluene	89	152	94	140	107	113	107	105
2-Amino-4-Nitrotoluene	89	97	88	111	87	118	94	103
o-Chlorotoluene	87	101	98	118	99	108	94	111
m-Chlorotoluene	97	107	99	110	101	104	103	104
p-Chlorotoluene	97	108	100	108	102	106	103	104
1-Chloro-2-Nitrobenzene	102	115	88	113	95	109	99	110
Hexachlorobenzene	117	133	94	131	99	116	104	113
Aniline	96	108	91	121	94	104	98	116

Project 3E161102BS09

BLAST OVERPRESSURE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6471	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISG'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
78 03 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3E161102BS09	00	001
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Medical Effects of Blast Overpressure: Basic Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

017100 Weapons Effects 002300 Biochemistry 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 03	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	8	450
FISCAL YEAR CURRENT 79	8	450

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: ALTSTATT, Leslie B., COL, MC
TELEPHONE: 202-576-3236

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Div of Med, Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: SANDER, Gary E., MAJ, MC
TELEPHONE: 202-576-3137
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: JAEGER, James, J., CPT, MSC
NAME: LORENZ, Patrick, CPT, MSC DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Blast Overpressure; (U) Pulmonary Hemorrhage; (U) Impulse Noise; (U) Biochemical Markers; (U) Pulmonary Physiology; (U) Acute Lung Injury

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define the physiological effects upon the human of blast overpressure generated by firing Army weapons systems in terms of: (a) the physical characteristics of the pressure wave responsible for injury; (b) the interaction between the wave and susceptible organs; (c) the threshold for injury of the various organ systems; and (d) potential means of prophylaxis and treatment of blast overpressure injury.

24. (U) Procedures include biochemical and physiologic measurements of pulmonary function; biochemical methods include radioimmunoassay of prostaglandins and coagulation by-products, measurement of coagulation parameters and complement activation changes in platelet function and number, and determination of changes in lung collagen content and structure induced by blast injury.

25. (U) 78 03 - 78 09. An animal model is being developed to study the biochemistry and physiology of acute lung injury. Hemorrhagic pulmonary edema will be created in dogs by intravenous injection of oleic acid. Decrements in pulmonary function will be assessed using a multiple gas rebreathing technique. An animal model for assessing inner ear damage (hair cell loss) induced by impulse noise is also under development.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3E161102BS09 BLAST OVERPRESSURE

Work Unit 001 Medical Effects of Blast Overpressure: Basic Studies

Investigators
Principal: MAJ Gary E. Sander, MC
Associates: CPT James J. Jaeger, MSC; Dr. Marvin Stein, Ph.D; CPT Patrick Lorenz, MSC; CPT Pritam Verma, MSC

Description

Studies are directed toward definition of the physiological effects upon the human of blast overpressure generated by firing Army weapons systems. Specific areas requiring investigation include: (1) the physical characteristics of the pressure wave responsible for injury; (2) the interaction between the blast wave and susceptible organs; (3) the threshold for injury of the various organ systems; and (4) potential means of prophylaxis and treatment of blast overpressure injury. In order to study the effects of the blast wave, it is necessary to simulate the wave utilizing a blast tube. This allows manipulation of the waveform and precludes dependence upon the availability of a weapon, charges, range, and artillery crew. Adequate detection of early tissue injury requires a basic science program aimed at identification of potential marker molecules and/or physiological procedures sufficiently sensitive to provide quantitation of such early tissue disruption.

Progress

1. Background Information

Blast overpressure induced injury occurs primarily in air-containing organs, specifically the nasal air sinuses, the lung, and the gastrointestinal tract. Of these, lung injury (hemorrhage and emphysema) is the most critical in that significant morbidity and mortality may result. Investigators at Lovelace Foundation in Albuquerque, New Mexico, are responsible for much of the existing information on blast injury (1-4). This work was largely supported by the Atomic Energy Commission and was directed at studying the effects of nuclear blast. In an attempt to estimate man's tolerance to air blast, these investigators exposed a number of species of animals to short (A-duration,

204 msec) and long (A-duration, 180-400 msec) duration air blast of progressively increasing peak pressures and determined percent mortality at each pressure level. When the data are expressed on a plot of log mortality vs. log pressure, each species falls along a straight line, and the straight lines themselves separate into two groups - a small animal group (mouse, rat, guinea pig, rabbit, hamster) and a large animal group (dog, sheep, goat, cattle). The common parameter in each group seems to be a nearly identical value for the normalized lung volume and the lung density. Since human lung volume and lung density fall in the group of large animals, man is assigned to the large animal group. If these data are then plotted as log LD50 (pressure dose producing 50% mortality) vs. log body mass for the species, a straight line results; this then allows an estimation of mortality for a species of any body weight. This produces only a general estimate, since an exact relationship between PSI and mortality does not exist. As an example, cattle, the heaviest of the large animals, was also the most susceptible to injury of the large animal group.

Mortality is an obvious endpoint - the animals dying shortly after single exposure are counted. Determination of less severe, non-fatal injury is much more elusive, and requires sacrifice of the animal and gross examination of the lungs for evidence of hemorrhage. The "threshold" of injury, or the earliest detectable stage of injury, is defined as that lowest pressure producing lung hemorrhage. The extent of lung hemorrhage generally parallels the pressure to which the animal is exposed, with petechial (pinpoint) hemorrhages gradually progressing to involve entire lung lobes with progressively higher exposure. This methodology is crude at best, requiring sacrifice of the animal, and producing only a single data point. Extrapolating from these animal data to man, the anticipated threshold for human lung injury would be between 30 and 40 PSI (3).

Three characteristics are required for an animal species to serve as a model for human injury. The species must be readily available, must lack animal protection groups, and must reasonably reproduce human physiology. Of available species, the sheep best fulfills these criteria.

Prof. C.J. Clemedson, the retired Surgeon General of Sweden, has also conducted extensive research in overpressure induced injury (5-8). His major contributions lie in the demonstration that frequency content of the pressure wave may be of critical importance in determining injury, in the description of the physiologic alterations following injury, and in the theoretical considerations of the mechanism of injury.

A small body of anecdotal data is available on human exposure to blast. During the OTII of the M198, crewmen were exposed to pressures of 2.9-3.7 PSI; two nosebleeds were reported but the severity of these was not recorded; an HEL study from November 1962 exposed volunteers to pressures up to 8 PSI to test the effectiveness of an ear plug. A foam rubber chest protector was required after unprotected troops complained of chest pain (9). The HEL director exposed himself to 7.5 PSI for ten exposures and reported coughing up blood. No definitive human exposure data exists on which to base injury estimates.

2. Requirements of the Program

The requirements are fourfold: (1) accurate simulation of the pressure wave; (2) a dose response curve for non-auditory injury; (3) a risk assessment based on the dose response curve; and (4) development of protective devices and techniques. A simulated waveform is necessary so that experimentation may be done without reliance upon weapon and charge availability. The ultimate requirement is the dose response curve, where the dose is the pressure wave and the response is the biological injury (or absence of injury). This will in turn require a more accurate knowledge of which parameters of the pressure wave are important (e.g., peak pressure, frequency) and a better means of evaluating biological injury. Once this dose response curve is established, then a risk assessment may be made for specific exposures - both in terms of the risk of a particular injury and the potential severity of that injury. This would then allow the line officer to make the final decision as to whether a particular risk is warranted. The final requirement is that ways of protecting crewmen be devised so that any risk of injury may be further reduced.

3. Review of the Current Status of the Blast Overpressure Program

a. Blast Wave Simulation

The major efforts of the non-auditory program have been directed toward simulating the M198 blast wave and toward initiating a basic research program to ellucide more effective means of detecting early lung injury. The blast wave simulation studies have been done in conjunction with the Lovelace Biomedical and Environmental Research Institute of Albuquerque, N.M., utilizing the blast tube facilities located there. By simultaneously detonating two primer cord charges at various distances on line with the open end of the tube, a waveform appearing very similar to that of the M198 with regard to time history and frequency content could be achieved for peak pressures of 9.2 PSI (190 dB),

5.2 PSI (185 dB), and 2.9 PSI (180 dB). After these simulations had been accomplished, sheep were exposed in groups of 10 to peak pressures of 190, 185, and 180 for 25 blasts occurring 20 seconds apart. An additional 10 sheep were exposed to a single blast at 185 dB. All animals were sacrificed immediately after exposure and examined for evidence of injury to the lungs, sinuses, and GI tract. Twenty-six control animals - animals from the same population but not exposed to blast - were also sacrificed. Hemorrhagic injury was found in 2/10 sheep at 190 dB; a single emphysematous bulla was found in one animal each of the 190/25, 185/25, and 185/1 groups. No such hemorrhagic or emphysematous lesions were found in the 180/25 exposure group or in any of the twenty-six control animals. Additionally, one focal GI tract hemorrhage was seen in the 190/25, as were several sinus hemorrhages in the 185/1, 185/25, and 190/25 groups. One unexplained sinus hemorrhage was detected in a control animal, but no GI tract lesions were detected. It is very important to remember that this sheep exposure study was performed solely as a preliminary exercise to allow the human volunteer study for validation of hearing protection to proceed. It must be emphasized that no animal injury was anticipated at pressures of less than 16-29 PSI and the findings described above were totally unexpected. The results of this study meant that the human study could not proceed until this apparent injury was better defined and either proved or disproved. THESE RESULTS HAD NO EFFECT UPON THE SAFETY RECOMMENDATIONS FOR THE FOE - THESE RECOMMENDATIONS STEMMED ENTIRELY FROM THE MIL-STD.

The totally unexpected outcome of the study led to the re-evaluation of the accuracy of the simulation as a possible explanation. Jaycor physicists were tasked with a detailed analysis of both real and simulated waveforms to determine if artifact had been introduced into the simulated wave. Early indications from this ongoing analysis are that the 190 dB simulated wave contained an excessive dynamic pressure component; furthermore, the technique of restraining the sheep during the 190 exposures may have contributed to the hemorrhagic injury seen. At present, the 185 dB exposures seem to represent a reasonable simulation, with only a small contribution from dynamic pressure. However, these dynamic pressure considerations have pointed out the failure of previous M198 testing to measure both static and dynamic components of pressure, and thus these field measurements must be made before a final decision on the adequacy of the simulation can be made.

b. Basic Research Program in the Biochemistry and Physiology of Acute Lung Injury

A suitable group of investigators have been assembled at WRAIR for initiation of the basic research program. A protocol entitled "Physiological and Biochemical Assessment of Acute Hemorrhagic Pulmonary Edema in the Dog" has been approved. This protocol has three principal objectives:

a. To develop an animal model of acute lung injury using dogs as the animal species and intravenous injection of oleic acid to produce injury. Oleic acid produces acute hemorrhagic pulmonary edema (10), one manifestation of air-blast induced lung injury. Thus the laboratory model is designed to simulate blast injury as closely as possible without resorting to actual blast exposure, which is not practicable in the laboratory setting.

b. To evaluate the sensitivity and specificity of a physiological technique which measures several important aspects of pulmonary function during the development of hemorrhagic pulmonary edema. Pulmonary tissue volume (edema fluid volume) will be determined by a technique (11) which measures the relative uptake of a soluble test gas during a rebreathing maneuver. This procedure, if successful, is ultimately applicable to human subjects.

c. To screen for potential blood-borne biochemical markers of injury. In order to be a useful marker of early injury, such a molecule must be established to originate from an alteration in lung physiology. Included in those molecules to be screened are the prostaglandin degradation products, prostacyclin, bradykinin, the angiotensins, and such potential indicators of early activation of coagulation such as complement binding to RBC, changes in factor VIII antigen to coagulant activity ratios, and early fibrinogen cross-linking.

4. The non-auditory requirements are fourfold: (1) the accurate simulation of the pressure wave; (2) a dose response curve for non-auditory injury; (3) a risk assessment based on the dose response curve; (4) development of protective devices and techniques. Work with the blast tube at Lovelace has resulted in simulated M198 blast waves which appear on paper to reproduce the actual waves quite accurately. The Jaycor analysis of these wave forms is still in progress but indications are that the 190 dB wave will need further revision. Attempts are now underway at Lovelace to reduce the dynamic component of this wave. The development of the dose response curve is dependent upon the proper definition of both the dose (the blast wave) and the response (biological injury). The overpressure program has a physicist actively investigating the physical parameters of the pressure wave, and the basic research program at WRAIR is directed toward the early detection of biological injury. It has been suggested that sheep be exposed to the M198/M203 in operator

positions to determine if non-auditory injury occurs in this setting. It is currently anticipated that this study will be delayed until Spring 1979 when the M203 charge problems are resolved and the RAM study proceeds again. This will allow piggy-backing of the sheep study and avoid the requirement for an additional 250 M203 charges. It is important to realize that this sheep study as proposed will not alter the current safety recommendations. This study will be subject to the same difficulties in interpretation that the existing study has been plagued with. It will provide only a single point on the dose response curve, and the results will not be directly applicable to humans. The risk assessment will be dependent upon the completion of the dose-response curve. Work with protective devices and techniques will begin as soon as the validation of the simulated waves has been completed. It is necessary to have a waveform of sufficient magnitude such as to reproducibly cause significant injury, such that protection or lack of protection can be reasonably determined without resorting to large numbers of animals.

Project 3E161102BS09 BLAST OVERPRESSURE

Work Unit 041 Medical Effects of Blast Overpressure: Basic Studies

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